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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/11, 15/63, 15/85, 15/86, 7/00, C07K 14/00

A1

(11) International Publication Number: WO 00/12693

(43) International Publication Date: 9 March 2000 (09.03.00)

(21) International Application Number: PCT/US99/19468

(22) International Filing Date: 26 August 1999 (26.08.99)

(30) Priority Data:
60/097,961 26 August 1998 (26.08.98) US
60/102,691 1 October 1998 (01.10.98) US

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(81) Designated States: AE, AL, AM, AT, AT (Utility model), AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, CZ (Utility model), DE, DE (Utility model), DK, DK (Utility model), EE, EE (Utility model), ES, FI, FI (Utility model), GB, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (Utility model), SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: DNA TRANSFER TECHNOLOGY FOR SHUTTLING LARGE INSERTS INTO MAMMALIAN CELLS

(57) Abstract

A recombinant plasmid useful for the production of large–insert episomes in mammalian and bacterial cells comprises a lymphotrophic herpes virus segment which (a) contains an origin of plasmid replication (oriP) and a heterologous origin of bacterial replication, and (b) is maintained as an episome in both bacterial and mammalian host cells; and a heterologous insert segment linked to the lymphotrophic herpes virus segment. The heterologous insert segment has a length of at least 50 kilobases.

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DNA TRANSFER TECHNOLOGY FOR SHUTTLING LARGE INSERTS INTO MAMMALIAN CELLS

This invention was made with Government support under grant number DE-FG05-91ER61135 from the DOE. The Government has certain rights to this invention.

Related Applications

This application claims priority from commonly owned, copending applications Serial No. 60/102,691, filed October 1, 1998, and Serial No. 60/097,961, filed August 26, 1998, the disclosures of which are to be incorporated by reference herein in their entirety.

Field of the Invention

This invention concerns vectors and methods that are useful for shuttling large DNA inserts from yeast and/or bacterial cells into mammalian cells.

Background of the Invention

Current gene therapy vectors suffer from various inherent limitations. Retroviral vectors depend on dividing cells and integrate randomly into the target cell genome with potential undesirable gene activation or inactivation effects (Naviaux and Verma, *Curr. Opin. Biotech,* **3**, 540 (1992)) . First generation adenoviral vectors are non-replicating, precluding their long-term persistence in dividing tissues (Crystal, *Science* **270**, 404 (1995)) . Depending on the sequences present in AAV-based vectors, they may have a preference for random integration rather than for specific integration into chromosome 19q (Kotin et al. *Proc. Natl. Acad. Sci. USA* 87, 2211 (1990); Shelling and Smith, *Gene Therapy* **1**, 165 (1994)) . In addition, all these

vectors are restricted in their cloning capacity in bacteria due to the limitations of the pBR322 backbone and in mammalian cells due to the packaging limit of the corresponding virus. Therefore, most genes are cloned into these vectors and expressed as cDNAs using "foreign" promoters instead of as genomic entities with their natural promoters and control elements in correct spatial orientation. Consequently, the expression level of the transduced genes is affected since the "optimal" promoter for regulating gene activity will depend on the host cell type (Dillon and Grosveld, *Curr. Opin. Genet. Dev.* **4**, 260 (1994)) . In addition, integration of the transduced gene into host chromosomes can surrender gene activity to position effects, and lead to promoter shut-off (Naviaux and Verma, *supra*).

Human DNA fragments in the large size range, i.e. 100 kb to 1 Mb, are panelable as sets of various libraries whose respective cloning vectors are based on the bacteriophage P1 (Ioannou et al. *Nature Genet.* **6**, 84 (1994); Shepherd et al., *Proc. Natl. Acad. Sci. USA* **91**, 2629 (1994)) , bacterial F1 factor (Kim et al., *Genomics* **34**, 213 (1996)) or budding yeast genomic elements (Burke et al., *Science* **236**, 806 (1987)) . In particular, yeast artificial chromosomes (YACs) allow cloning of DNA fragments of more than 1 Mb. Genes delivered as YAC clones can be functional *in vitro* (Huxley et al., *Genomics* **9**, 742 (1991); Peterson, Zitnik et al., *Proc. Natl. Acad Sci. USA* **90**, 11207 (1993a)) and in transgenic mice (Schedl et al., *Nature* **362**, 258 (1985); Jakobovits et al., *Nature* **362**, 255 (1993); Peterson, Clegg, et al., *Proc. Natl. Acad. Sci. USA* **90**, 7593 (1993); Peterson et al., *Trends Genet.* **13**, 61 (1997)) . For example, the β-globin multigene family with the LCR and downstream control elements can be delivered in its natural chromatin configuration. Hence, high level expression can be obtained independently of the integration site into host cell chromosomes (Peterson, Zitnik et al., *supra*; Peterson, Clegg et al., *supra*). However, DNA transfer efficiencies are low due to the large size of the YACs, and YACs integrate randomly in the vast majority of cases making gene expression subject to position effects. In addition, integration of large YACs is rather inefficient and frequently associated with rearrangements and deletions.

The delivery of a gene of interest as an episome offers a potential solution to eliminate position effects and alleviate size constraints. However, such a strategy requires coordination of episomal replication with the cell cycle and equal distribution of newly-replicated episomes between daughter cells, to avoid progressive dilution of

the transduced gene in dividing cells. The latent episomal Epstein-Barr virus (EBV) has been shown to contain such replication and segregation activities (Yates et al., *Nature* **313**, 812 (1985)) . Its latent origin of replication, oriP, fulfills both functions through interaction with the unique viral transactivator EBNA-1. EBNA-1 binds specifically to oriP at two different sites (Rawlins et al., *Cell* **42**, 859 (1985)) . Episomal replication is initiated at the dyad symmetry element of oriP (Gahn and Schildkraut, *Cell* **58**, 527 (1989)) while retention of the replicated episomes in the nucleus occurs through the family of repeats (Yates, In *DNA Replication in Eukaryotic Cells*, 751 (M. Pamphilis, ed. 1996)). Engineered oriP containing plasmids with a cloning capacity of up to 350 kb can be maintained in human cells expressing EBNA-1 as episomes (Sun et al., *Nature Genet.* **8**, 33 (1994)).

U.S. Patent No. 5,275,942 to Vos describes a recombinant plasmid useful for the production of large insert, stably maintained episomes in mammalian cells. The plasmids comprise a lymphotrophic herpes virus segment containing an origin of plasmid replication. The plasmids are maintained as episomes in mammalian cells. Although the plasmids include a bacterial origin of replication, they cannot be maintained as episomes in bacterial cells. The ability to maintain such plasmids as episomes in bacterial cells is, however, extremely desireable. *See also* Sedimenti and Calos, *Adv. Drug Dev. Rev.* **30:** 13 (1998); M. Calos, U.S. Patent No. 5,707,830.

Summary of the Invention

A first aspect of the present invention is a recombinant plasmid useful for the production of large-insert episomes in mammalian and bacterial cells. The plasmid comprises a lymphotrophic herpes virus segment which (a) contains an origin of plasmid replication (oriP) and a heterologous origin of bacterial replication, and (b) is maintained as an episome in both bacterial and mammalian host cells; and a heterologous insert segment linked to the lymphotrophic herpes virus segment, the heterologous insert segment having a length of at least about 50 kilobases.

A second aspect of the present invention is a method for transforming mammalian cells. The method comprises: transfecting a mammalian cell with a recombinant plasmid as described above.

A third aspect of the present invention is a transformed cell (preferably a bacterial, yeast, or mammalian cell) containing a recombinant plasmid, the

recombinant plasmid comprising: a lymphotrophic herpes virus segment which (a) contains an origin of plasmid replication (oriP) and a heterologous origin of bacterial replication, and (b) is maintained as an episome in both bacterial and mammalian cells; and a heterologous insert segment linked to the lymphotrophic herpes virus segment, the heterologous insert segment having a length of at least about 50 kilobases.

A further aspect of the present invention is a large insert DNA library comprising a plurality of transformed cells (preferably mammalian, bacterial or yeast cells), each of the transformed mammalian cells containing a recombinant plasmid, the recombinant plasmid comprising: a lymphotrophic herpes virus segment which (a) contains an origin of plasmid replication (oriP) and a heterologous origin of bacterial replication, and (b) is maintained as an episome in both bacterial and mammalian host cells; and a heterologous insert segment linked to the lymphotrophic herpes virus segment, the heterologous insert segment having a length of at least 50 kilobases and comprising a member of the DNA library.

A further aspect of the invention is a large-insert DNA library comprising a plurality of infectious lymphotrophic herpes virus virions, each of the virions containing a recombinant DNA molecule, the recombinant DNA molecule comprising: a lymphotrophic herpes virus segment which is capable of infecting mammalian cells, is maintained as an episome therein, and produces infectious virions in a suitable host; and a heterologous insert segment linked to the lymphotrophic herpes virus segment, the heterologous insert segment having a length of at least 50 kilobases and comprising a member of the DNA library; the lymphotrophic herpes virus segment having regions deleted so that the recombinant DNA sequence retains the capability of producing infectious virions in a suitable host; the lymphotrophic herpes virus segment including an origin of plasmid replication (oriP), a heterologous origin of bacterial replication, a lytic origin of replication (oriLyt), and long terminal repeat regions (TR).

A particular aspect of the present invention is a recombinant plasmid useful for the production of large-insert episomes in mammalian cells, comprising: an Epstein-Barr virus segment containing an origin of plasmid replication (oriP), a lytic origin of replication (oriLyt), a heterologous origin of bacterial replication and a fused long terminal repeat region (TR); and a heterologous insert segment linked to the

Epstein-Barr virus segment, the heterologous insert segment having a length of at least about 50 kilobases.

A further aspect of the present invention is an infectious lymphotrophic herpes virus virion containing a recombinant DNA molecule, the recombinant DNA molecule comprising: a lymphotrophic herpes virus segment which is capable of infecting mammalian cells, is maintained as an episome therein, and produces infectious virions in a suitable host; and a heterologous insert segment linked to the lymphotrophic herpes virus segment, the heterologous insert segment having a length of at least about 50 kilobases; the lymphotrophic herpes virus segment having regions deleted so that the recombinant DNA sequence retains the capability of producing infectious virions in a suitable host; the lymphotrophic herpes virus segment including an origin of plasmid replication (oriP), a heterologous origin of bacterial replication, a lytic origin of replication (oriLyt), and long terminal repeat regions (TR).

A further aspect of the present invention is a method of introducing a DNA of interest into a mammalian subject, comprising: directly or indirectly administering a vector selected from the group consisting of plasmids as described above or virions as described above in an amount effective to introduce the DNA of interest.

A further aspect of the present invention is a transgenic non-human mammal comprising cells containing a plasmid as described above, or transfected with a virion as described above.

A further aspect of the present invention is a method of producing a heterologous protein in a host cell, comprising the steps of: transfecting the host cell with a vector selected from the group consisting of plasmids as described above and virions as described above, wherein the heterologous insert encodes and expresses the heterologous protein in the host cell; and expressing the heterologous protein in the host cell.

Brief Description of the Drawings

Figure 1A: BAC HAEC Strategy. Schematic map of the BAC-HAEC vector pBH140. Inserts are introduced into the NotI sites of the multiple cloning site (MCS). The hygromycin resistance gene allows for positive selection of BAC-HAEC transduced mammalian cells. OriP and the CMV driven EBNA-1 gene enable episomal maintenance in human cells. EBNA-1 as well as the negative selection

marker HSV-tk can be eliminated by cre/loxP mediated pop-out recombination and replaced by other elements (e.g. circular YAC components) by the cre mediated pop-in reaction. Par A, Par B and repE are the BAC elements and allow stable maintenance of large inserts in bacteria.

Figure 1B: Cloning flow-chart into the BAC-HAEC vector. BAC/P1/PAC libraries are screened by PCR with locus-specific primers. Positive clones are digested with NotI (pop-out). The large inserts are purified by pulse field gel electrophoresis on agarose gels and ligated into NotI-digested dephosphorylated linear pBH140 vector DNA (pop-in). The derived BAC-HAEC clones are lipofected into human cells and the resulting hygromycin resistant cell transformants are analyzed for episomal stability, copy number and gene expression (see text for details).

Figure 1C : NotI analysis of bacterial BAC, PAC and BAC-HAEC clones by pulsed-field gel electrophoresis and ethidium bromide staining: lane 1 : l /HinDIII marker ; lane 2 : 50 kb l ladder ; lane 3 : 185 kb human β-globin BAC-HAEC clone p148BH ; lanes 4-6 : BACs with anonymous human DNA of 180 kb, 140 kb, 250 kb inserts, respectively; insert doublets of linear DNA (lanes 3-8) and weak bands of closed circular DNA (lanes 3-4) are due to partial NotI digestion; lanes 7-8 : PAC clone with a 170 kb human HPRT insert; the asterisk * at the bottom of the gel corresponds to the various vector bands, whose positions of migration vary slightly due to size differences (lane 3: 16.9 kb pBH140; lanes 4-6: 7 kb p108L; lane 7-8: 17 kb CYPAC).

Figure 2A: Episomal gel analysis of the BAC-HAEC human β-globin clone in human cell transformants: Shuttling of 180 kb BAC-HAEC from bacteria to human cells: Episomes isolated from D98/Raji~p148BH cells were run uncut on a 0.8% agarose gel and blotted onto a nylon membrane ; the filter was probed with the vector specific fragment hyg-i (left/panel) or with the EBV specific probe Bam W (right/panel); lane 1 : 140 kb bacterial marker (anonymous human BAC-HAEC clone) ; lane 2 : 180 kb bacterial marker (anonymous human BAC-HAEC clone) ; lane 3 : p148BH DNA isolated from bacteria; lane 4 : blank ; lane 5 : episomal DNA isolated from a pool of approx. 50 D98-Raji human cell transformants; the asterisk * at the bottom of the gel (left panel) indicates the presence of linear DNA due to breakage of circular episomal DNA during sample purification.

Figure 2B: Long-term stability of the 180 kb BAC-HAEC in human cells. Comparative episomal gel analysis was performed on the 185 kb circular human ß-globin BAC-HAEC DNA and a 180 kb anonymous BAC-HAEC clone after one year of continuous culture of the D98-Raji cell transformants. Episomes isolated from the hygromycin D98-Raji cells were run uncut on a 0.8 % agarose gel, blotted onto nylon membranes and the filters were probed with either the vector specific hyg-i fragment (left/panel), with an oriP-EBNA 1 fragment detecting both HAEC and EBV DNA (middle/panel) or Bam W fragment detecting only the Raji EBV genome (right/panel) or . Lane 1: 180 kb anonymous BAC-HAEC episomes ; lane 2 : p148BH human BAC-HAEC β-globin episomes; c : intact circular DNA; l : broken linear DNA .

Figure 3 : FISH analysis of BAC-HAEC human β-globin DNA in human cell tranformants. Metaphase and interphase spreads from parental (A, B) and transformed (C, D) D98-Raji cells were probed with a HAEC specific probe, pBeloBac11. The FISH technique as applied to metaphase chromosomal spreads distinguishes integrated and episomal sequences as close double labels localized on sister chromatid and individual hybridization signals, respectively (Sun et al. 1996) .

Figure 3E: Quantification of the number of ß-globin BAC-HAECs per nucleus in D98-Raji cells stably transformed with p148BH. Individual FISH signals from 50 metaphase and interphase spreads of controls and transformed D98-Raji cells were hybridized with the HAEC specific probe pcH110 and counted as described (Banerjee et al., *Nature Med.* I, 1303 (1995)).

Figure 4A: Expression analysis of human β-globin from the BAC-HAEC established in human cells. RT PCR assays on RNA isolated from one year old cultures of BAC-HAEC D98/Raji cell transformants and fresh human blood cells. RT PCR on total cellular RNA was accomplished using ß-globin specific primers as previously described (Sierakowska et al., *Proc. Natl. Acad. Sci. USA* **93**, 12840 (1996)). Lanes 1, 10-11 : control red blood cells (B); lane 2-5 : D98-Raji~p148BH cells; lanes 6-7: parental D98-Raji cells ; lanes 8-9 : D98-Raji~p148BH cells. Reaction conditions were as follows. Lanes 2, 3 and 10: no RT reaction. Lanes 2-5: 1 ul RT-PCR template; lanes 6-9; 3 ul RT-PCR template. Lanes 2, 4, 6, 8 and 11: 18 PCR cycles; lanes 1, 3, 5, 7 and 9-10: 20 PCR cycles. The same reaction volumes were loaded, except 1/100x volume for control blood samples.

Figure 4B. Comparative RT PCR levels of ß-globin expression from extrachromosomal BAC-HAEC and stably integrated CMV-driven minigenes in human cell transformants. RT PCR on total cellular RNA was performed as indicated above. Lanes 1-4: D98-Raji~p148BH cells; lanes 5-6: parental D98-Raji cells; lanes 7-12: HeLa clones expressing a CMV-driven human ß-globin gene with lanes 7-8: HeLa cell clones BH, lanes 9-10: HeLa clone A and lanes 11-12: HeLa clone B (Sierakowska et al., *supra*). Reaction conditions were as follows. Lanes 1 and 3: no RT reaction. Lanes 3, 4, 6, 8, 10, 12: 1 ul RT-PCR template; lanes 1, 2, 5, 7, 9, 11; 3 ul RT-PCR template. All samples were amplified with 18 cycles and the same reaction volumes were loaded in all lanes.

Figure 5. Comparison of the amino acid changes between different EBNA1 proteins. The numbers denote the coordinates of the B95.8 EBNA1 sequence. 15 amino acid differences were found in the Raji EBNA1 protein sequence as compared to the B95.8 derived EBNA1. NPC and C15 derived EBNA1 protein sequences are included (Snudden et al., *Oncogene* **10**, 545 (1995)). The GlyAla domain encompasses amino acids 91 to 358 of the B95.8 EBNA1. The amino acid differences from 471 to 492 fall within a putative DNA binding domain, and amino acids differences 502 to 594 fall within two regions thought to be involved in EBNA1 dimerization(Chen et al., *J. Virol.* **67**, 4875 (1993)). The (*) symbol at # 471 indicates a potentially important amino acid change (see text). The (-) symbol indicates no change relative to the B95.8 EBNA1 sequence.

Figure 6. Schematic representation of the 20.3 kb oriP-based plasmid pH2î1 with the various EBNA1 expression cassettes inserted at the unique HindIII site. Expression of the EBNA1 genes is driven by the cytomegalovirus immediate early promoter (CMV). The minimal EBV *cis*-sequences are: EBV TR, terminal repeat sequence; EBV oriP, latent origin of plasmid replication; EBV orilyt, origin of lytic replication. The marker lacZ gene is driven by the SV40 promoter and the hygromycin phosphotransferase gene (hygromycin^r) is driven by the HSV thymidine kinase (TK) gene promoter. pH211 is identical to pH210 (Banerjee et al., *supra* (1995)). except for the inverted orientation of the SV40-driven lacZ gene. The schematic diagram of the expression cassettes for the different EBNA1 isoforms is also shown. The black box represents the variably sized GlyAla domain of each

EBNA1 protein. The approximate size of each cassette is indicated above each construct. The difference in length of each GlyAla domain is indicated by a shortened box. The CMV promoter is indicated by the open box at the 5' end of each cassette. The B95.8 EBNA1 coding region was swapped with the Raji EBNA1 coding region at the AvrII and SacII sites to create the expression cassette for CMVRaji.E1. Likewise, AvrII to SacII restriction fragment of the EBNA1 gene in the plasmid pREP7 (Invitrogen) was inserted into the same sites in the B95.8 EBNA1 construct to create the expression cassette for the truncated IR3del clone (CMVIR3del.E1). The polyadenylation signal (@60 bp downstream) is the endogenous EBNA1 site used by EBV.

Figure 7A. Southern blot analysis of 23.0 kb OriP episomes in short-term cultures. Equal amounts of each plasmid (10ug) were transfected into DG75 cells and pools of stable cell transformants were obtained by selection in the presence of hygromycin. An alkaline lysis procedure was used to extract extrachromosomal DNA from each pool one month post-transfection. Plasmids were resolved by PFGE. Blots were analyzed with an ^{32}P labeled EBNA1 specific probe. Each transformed clone is indicated in subsequent lanes at the top of the autoradiogram. Linearized CMVB95.8 plasmid (Fig.6) was used as a molecular size standard in lane 4.

Figure 7B. Western blot analysis of EBNA1 expression in short-term cultures. Equal amounts of total protein extracted from the transformed DG75 cells were resolved by 7.5% SDS-PAGE. The blot was probed with an EBNA1 specific primary antibody[21] followed by goat antimouse secondary antibody provided in the ECL Western blot detection kit (Amersham). Molecular weights are shown to the right of the left panel. To illustrate quantitative differences in protein expression a lighter exposure of the blot is shown in the right panel.

Figure 7C. Short-term lacZ transgene expression assay. Approximately $2x10^6$ cells from one month stable cultures were lysed in the presence of the substrate O-nitrophenyl-b-D-galactopyranoside (ONPG). After more than 2 hours at 37°C the relative lacZ activity was measured by determining the optical density (OD) of the lysates at 420 nm. Negative controls are the untransfected parental DG75 cells and pools of DG75 cells transformed with the pH211 without EBNA1 (Fig.6). The

positive control is the EBNA1-expressing Raji cell transformed with episomal pH210 (Fig.6).

Figure 8A. Southern blot analysis of oriP episomes in long-term cultures. As in Figure 2A episomes were prepared from cultures grown continuously for 5 months and resolved by PFGE. Each pooled transformant is indicated at the top of each lane. A ^{32}P labeled EBNA1 specific probe was hybridized to the blot. The molecular size standard, linearized pCMVB95.8 EBNA1 plasmid, is in lane 4.

Figure 8B. Comparative Western blot analysis of EBNA1 expression in both short-term and long-term cultures. Total protein from both one month (lanes 1,2,3) and five month (lanes 6,7,8) cultures were resolved by 7.5% SDS-PAGE and EBNA1 expression was detected as in Figure 2B. Each EBNA1 expressing clone is indicated at the top of each lane. The EBNA1-expressing positive control RajipH210 was included (lane 4), as well as the EBNA1-negative control DG75 (lane 5). The Rainbow molecular weight markers are indicated in lane 9, and the molecular weights are shown to the right of the autoradiogram.

Figure 8C. Graphical analysis of quantitative loss of transgene expression in long-term cultures. The beta-gal activity from each plasmid at various time intervals was normalized to the initial level. The betal-gal levels of each transformant were estimated as in Figure 2C. The relative transgene expression level at each time point was then plotted in graphical form on a semilog scale to highlight the differential loss with the different EBNA1 expressing plasmids. The time points per month were plotted on the x-axis and the normalized beta-gal activities were plotted on the y-axis (, ,).

Figure 8D. Differential loss of lacZ gene activity in BJAB and HH514 cells. CMVRaji.E1 and CMVIR3del.E1 expression plasmids were transfected (10ug) into BJAB and HH514 cells. Hygromycin resistant pools were grown continuously for 3.5 months in culture. Each clone is indicated below the histogram. LacZ gene activity was then determined as above.

Figure 9A. Long-term quantitative Southern analysis of oriP episomes. Transformed cells were grown continuously for up to 6 months post-transfection.

Episomal extracts were prepared from the pooled transformants at 1 month time intervals, linearized with HindIII and resolved by PFGE. The blot was co-hybridized with ^{32}P labeled probes specific for the episomal oriP plasmids (EBNA1 gene) and human mitochondrial (huMit) DNA. Time intervals are indicated under the figure. Starting at lane 2, each group of 3 lanes represents samples derived from CMVB95.8.E1, CMVRaji.E1, and CMVIR3del.E1 transformants every month over half a year, respectively. The top band is the signal derived from the 20.3 kb pH211 backbone of the expression cassettes; the bottom band is the signal derived from the 5.0 kb HindIII fragment of huMit DNA. The faint signal just below the pH211 band is a nonspecific for the huMit probe. Lane 1 is pH211 (EBNA1-negative) transfected DG75 as non-episomal control. Lane 20 is untransfected parental DG75. Lane 21 is 100 picograms of linearized pCMVB95.8 DNA as episomal copy number standard.

Figure 9B. Graphical analysis of quantitative loss of episomal oriP/EBNA1 plasmids in long-term cultures. From Figure 9A, the signal from each oriP plasmid in each time interval was normalized to the signal from its huMit internal standard. The plasmid copy number of each transformant was then estimated from the intensity of the signal from the copy number control plasmid LpH211 in lane 21 of Figure 9A. The plasmid copy number in each time point is plotted in graphical form on a semilog scale to show the differential loss of the different EBNA1 expressing plasmids. Time points in months are plotted on the x-axis and the copy number are plotted on the y-axis.

Detailed Description of Preferred Embodiments

The present invention may be made and used as described in U.S. Patent No. 5,275,942 to Vos. the disclosure of which is incorporated by reference herein in its entirety. Thus, the lymphotrophic herpes virus segment employed herein is preferably capable of producing infectious virions in a suitable host cell, and the lymphotrophic herpes virus segment may have regions deleted so that the recombinant plasmid retains the capability of producing infectious virions in the host cell. For large inserts, however, it is not critical that the capability of producing infectious virions is

retained, as a desireable feature of the invention is the ability of producing episomes in the desired host cells. The heterologous insert segment may include a centromere operable in the host cell, particularly mammalian cells. In a preferred embodiment, the lymphotrophic herpes virus segment comprises an Epstein-Barr virus segment. If desired, the lymphotrophic herpes virus segment has regions deleted which render the plasmid capable of producing infectious virions only in a host cell containing helper sequences.

The origin of bacterial replication is one that maintains the plasmid as an episome in a bacterial cell such as an *Escherichia coli* host cell. An example is an F origin from *Escherichia coli*. This aspect of the invention may be carried out as described in U.S. Patent No. 5,733,744 to Hamilton, titled *Binary BAC Vector*, the disclosure of which is incorporated by reference herein in its entirety.

Heterologous inserts used herein are preferably large inserts of about 50, 100, 150 or 200 kilobases (Kb; thousand bases) up to about 1, 2, 3 or 4 megabases (Mb; million bases) or more. While the invention is described above with reference to inserts of at least about 50 Kb, it will be appreciated that in certain instances the invention may be practiced with smaller inserts.

The term "host cell" as used herein may be a bacterial, yeast, or mammalian cell. Host cells may be transformed with the plasmid and virion vectors of the invention for any purpose, including the expression of markers therein, the creation of a library therein, the expression of a heterologous protein therein (for fermentation production of a protein, animal production of a protein, gene therapy, expression of a marker protein to study expression patterns, etc.).

The term "bacterial cell" as used herein is intended to include any bacterial cell, with *Escherichia coli* preferred.

The term "mammalian" as used herein with respect to cells or subjects refers to any mammalian species, including human, goat, sheep, cow, rat, mouse, dog, cat, etc. Human is preferred.

The term "episome" as used herein refers to a heterologous DNA that is stably maintained in a host cell and is hereditable to progeny cells. The episome is preferably circular and can be maintained as a single copy in the host cell (though it may be induced to high copy number if desired).

The phrase "regions deleted" as used herein has its conventional meaning, is not limited to particular functional or structural regions, and simply refers to the deletion of sufficient portions of nucleic acid to allow the insertion of the heterologous insert, or the imparting of the indicated function.

The plasmids and constructs of the invention may be further modified by inserting a heterologous origin of yeast replication such that the plasmid is also maintained as an episome in yeast cells. Such inserts are known in the art of Yeast Artificial Chromosomes (YACs), and may be obtained in accordance with known techniques. The inserts described in U.S. Patents Nos. 5,776,773 and 5,643,763 (the disclosures of which are incorporated herein by reference in their entirety) may be used for this purpose.

As noted above, the invention provides a method for transforming mammalian cells. The method comprises transfecting a mammalian cell with a recombinant plasmid as described above. The mammalian cells may be grown as a monolayer in in vitro cell culture, and wherein the transfecting step is carried out by lipofection. In one embodiment, the mammalian cell is a B-lymphoblastoid cell; in another embodiment, the mammalian cell is a fusion of a mammalian epithelial cell and a mammalian B-lymphoblastoid cell. The mammalian cell may be an epithelial cell; the mammalian cell may or may not be one capable of producing infectious virions from the recombinant plasmid. Cells so transformed may be grown as a monolayer in in vitro cell culture.

When the invention is used to create a large insert library, the library may be a human genomic DNA library, and cells may be human cells. Genomic libraries created by the invention may be partial or complete genomic libraries.

As noted above, a further aspect of the present invention is a method of introducing a DNA of interest into a mammalian subject, comprising directly or indirectly administering administering a vector selected from the group consisting of plasmids as described above or virions as described above in an amount effective to introduce the DNA of interest. Direct injection may be by any suitable means, such as by parenteral injection (e.g., intraveneous injection) of the virus. Indirect administration may be carried out by any suitable means, such as by introducing the DNA into a cell such as a mammalian hematopoietic stem cell or embryonic stem cell *ex vivo* and then administering the cell so transformed to the subject.

A further aspect of the present invention is a transgenic non-human mammal comprising cells containing a plasmid as described above, or transfected with a virion as described above.

A further aspect of the present invention is a method of producing a heterologous protein in a host cell, comprising the steps of: transfecting the host cell with a vector selected from the group consisting of plasmids as described above and virions as described above, wherein the heterologous insert encodes and expresses the heterologous protein in the host cell; and expressing the heterologous protein in the host cell. Transfection and expression may be carried out in accordance with known techniques. The host cell may be a bacterial, yeast, or mammalian host cell. Any protein of therapeutic or industrial interest may be produced by such a technique, including but not limited to erythropoietin, insulin, interleukin-2, etc.

Where the invention is used for gene therapy, the heterologous insert may encode a therapeutic agent. Such therapeutic agents include, but are not limited to, nucleic acid sequences encoding tumor necrosis factor (TNF) genes, such as TNF-alpha; genes encoding inteferons such as Interferon- alpha , Interferon- beta , and Interferon-gamma ; genes encoding interleukins such as IL-1, IL-1 beta , and Interleukins 2 through 15; genes encoding G-CSF, M-CSF, and GM-CSF; genes encoding adenosine deaminase, or ADA; the Zap70 kinase gene; genes which encode cellular growth factors, such as lymphokines, which are growth factors for lymphocytes; the glucocerebrosidase gene; genes encoding epidermal growth factor (EGF), and keratinocyte growth factor (KGF); genes encoding soluble CD4; the beta -globin gene; Factor VIII; Factor IX; T-cell receptors; the alpha -iduronidase gene; the LDL receptor, ApoE, ApoC, ApoAI and other genes involved in cholesterol transport and metabolism; the alpha-1 antitrypsin (alpha 1AT) gene; the ornithine transcarbamylase (OTC) gene; the CFTR gene; the insulin gene; suicide genes such as, for example, viral thymidine kinase genes, such as the Herpes Simplex Virus thymidine kinase gene, the cytomegalovirus virus thymidine kinase gene, and the varicella-zoster virus thymidine kinase gene; Fc receptors for antigen-binding domains of antibodies; antisense sequences which inhibit viral replication, such as antisense sequences which inhibit replication of hepatitis B or hepatitis non-A non-B virus; antisense c-myb oligonucleotides; multidrug resistance genes such as the MDR-1 gene; and antioxidants such as, but not limited to, manganese superoxide dismutase

(Mn-SOD), catalase, copper-zinc-superoxide dismutase (CuZn-SOD), extracellular superoxide dismutase (EC-SOD), and glutathione reductase; and selectable markers such as the neomycin resistance (neoR) gene, the beta -galactosidase (lacZ) gene, the chloramphenicol transferase (CAT) gene, and the NGF-R gene.

The nucleic acid sequence encoding at least one therapeutic agent as described above, or other protein to be expressed in the host cell, is under the control of a suitable promoter. Suitable promoters which may be employed include, but are not limited to, adenoviral promoters, such as the adenoviral major late promoter; or heterologous promoters, such as the cytomegalovirus (CMV) promoter; the respiratory syncytial virus (RSV) promoter; inducible promoters, such as the MMT promoter, the metallothionein promoter; heat shock promoters; the albumin promoter; the ApoAI promoter; human globin promoters; viral thymidine kinase promoters, such as the Herpes Simplex thymidine kinase promoter; retroviral LTRs (including the modified retroviral LTRs hereinabove described); the beta -actin promoter; and human growth hormone promoters. The promoter also may be the native promoter which controls the gene encoding the therapeutic agent. It is to be understood, however, that the scope of the present invention is not to be limited to specific foreign genes or promoters.

The plasmid vector described herein may transduce host cells through any means known in the art. Such means include, but are not limited to, electroporation, the use of liposomes, such as hereinabove described, and $CaPO_4$ precipitation. In one alternative, the plasmid may be encapsulated into a liposome, or coupled to a lipid, and then administered to a host, also as herein described.

When administered in vivo, the viral particles are administered to a host in an amount effective to produce a therapeutic effect in a host. In general, the particles are administered in an amount of from 1 to about 10 particles per cell. The exact dosage of particles to be administered is dependent upon the factors hereinabove described.

a. EBNA-1 Proteins.

In the present invention, the vector prefererably further comprises a copy of an EBV Nuclear Antigen-1 (EBNA-1) gene that can be functionally expressed in a mammalian cell. Any nucleic acid encoding an EBNA-1 gene can be employed that facilitates the stable maintenance of the vector in the host cell. Suitable EBNA-1

genes include those described in U.S. Patent No. 5,707,830 to Calos, the disclosure of which is incorporated herein by reference. Preferably, the EBNA-1 gene includes a partial IR3 domain deletion, such that the IR3 domain is 200, 300, 400 or 450 nucleotides in length to 550, 600, 700, or 750 nucleotides in length. A particularly suitable EBNA-1 gene is the Raji EBNA-1 gene, described in further detail below.

An additional aspect of the present invention is an isolated nucleic acid encoding an EBV Nuclear Antigen-1 (EBNA-1) gene that has a partial IR3 domain deletion, wherein said IR3 region is from 300 to 700 nucleotides in length, along with vectors such as plasmid vectors containing the same. The encoded proteins are useful for facilitating the stable maintenance of vectors containing and expressing the protein in host cells. Thus, such nucleic acids can be employed in all the vectors, methods, and products disclosed in U.S. Patent No. 5,275,942 to Vos (the disclosure of which is incorporated herein by reference) with heterologous insert segments great than 100 kb as described therein, or with heterologous insert segments less than 100 kb.

The present invention is explained in greater detail in the following non-limiting examples.

EXAMPLE 1

A System for Shuttling 200 KB Bac/Pac Clones Into Human Human Cells: Stable Extrachromosomal Persistence and Long-Term Ectopic Gene Activation

Recently, an oriP-containing vector system based on homologous recombination in budding yeast has been developed for shuttling large genomic inserts from a YAC library into EBNA-1 positive human cells (Simpson et al., *Mol. Cell. Biol.* 16, 5117 (1996)). Here an alternative strategy based on simple and standard cloning techniques and a second generation HAEC vector is presented (Sun et al., *supra* 1994) . This vector pBH140 has a large cloning capacity because of its F1 based origin of replication, a situation similar to the BAC/P1 vectors. In addition, it contains the EBV latent origin *oriP* and the EBNA-1 gene allowing episomal maintenance in human cells. The multiple cloning site of pBH140 with its rare cutter restriction sites allows the isolation of large genomic inserts from pre-existing libraries such as P1, PAC, BAC as well as YACs. In the present study, it was investigated whether the hybrid BAC-HAEC vector pBH140 allowed the

establishment and maintainenance of large human genomic inserts as stable episomes in human cells and whether the gene of interest was expressed over an extended cultivation period. As a model, a 200 kb human β-globin cluster genomic fragment was chosen.

A. MATERIALS AND METHODS

Construction of BAC-HAEC clones. A human β-globin genomic clone with an insert size of 185 kB was obtained by PCR screening from a human genomic (PAC) library (Ioannou et al., *supra* 1994) . This large PAC clone p148 contained the 5' and 3' end of the human β-globin cluster including the Locus Control Region, LCR (P. Ioannou, pers. comm.). The 185 kb insert was removed by NotI digestion, purified on a pulsed field gel (Biorad CHEF Mapper) and subcloned into Not-digested and alkaline phosphatase-treated BAC-HAEC vector pBH140 to give the β-globin BAC-HAEC clone (p148BH **Fig. 1**). BAC, PAC and BAC-HAEC DNA was isolated from 3ml overnight cultures by alkaline lysis, digested with NotI for several hours at 37° C and run on a 1.0% agarose gel in 0.5 x TBE at 14° C using the Biorad CHEF mapper under separation conditions for fragments between 6.0 kb and 300 kb in size.

Cell transformation. For transfection into human cells, the BAC-HAEC DNA was purified from 1 liter bacterial cultures and purified over Qiagen columns. 2.5 ug of BAC-HAEC plasmid DNA containing a 185 kb insert from the human β-globin cluster or 180 kb anonymous human DNA was transfected into 10^6/plate D98/Raji (DR) cells (Glaser and Nonoyama, *J. Virol.* **14**, 174 (1974)) with 10 ug lipofectamin (Gibco-BRL) as described (Sun et al. *supra* 1994) . Hygromycin selection (200 mg/ml) was started three days later and the cells were grown in E-MEM with 10% FBS in 5% CO_2 at 37° C. Resistant colonies became visible after 10 days and were allowed to grow as pools. All subsequent analyses were done on these pools.

Episomal gels. DNA from 6 x 10^7 stably transformed DR cells was isolated as previously described (Sun et al., *supra* 1994) . Briefly, the cells were lysed in 50 mM NaCl, 8 mM EDTA, 1% SDS, pH 12.5 by vigorous vortexing for two minutes and by subsequent incubation at 30° C for 30 minutes. Per 100 ml of lysis solution, 20

ml Tris (pH 7.0), 13.2 ml 5M NaCl and 1.2 ml 10 mg/ml proteinase K were added. After 30 minutes incubation at 37° C, the episomes were purified by four phenol extraction steps (phenol saturated with 0.2 M Tris (pH 8.0), 0.2 M NaCl) and one chloroform : isoamyl alcohol (24 : 1) extraction step. The DNA was precipitated with isopropanol at -20° C overnight and dissolved in 10 mM Tris-HCl, 1 mM EDTA(pH 8.0). Episomes from 1 x 10^7 cells were loaded per well on an 0.8% agarose gel and run unrestricted at 190 Volts, 4° C for 12 hours in 1 x TBE buffer. After ethidium bromide staining the DNA was nicked with 60 mJ in a Biorad GS Linker UV Chamber and blotted onto nylon membranes in 0.5 M NaOH / 1.5 M NaCl over night.The DNA was UV-cross-linked to the filters which were neutralized in 1.5 M NaCl, 0.5 M Tris (pH 7.8) and prehybridized for 2 hours at 42° C in a solution containing 50% formamide, 20 X Denhardt's, 6 x SSPE, 1 % SDS and 200 mg/ml single stranded salmon sperm DNA. Probes were ^{32}P-labelled by random priming for 2 hours at room temperature, denatured and added to the filters. After hybridization at 42° C overnight, the filters were washed 2 x 20 minutes in 0.5 x SSC, 0.1 % SDS at 50° C and exposed to Kodak X-OMAT X-ray films using an intensifying screen at -80° C. For size determination of the episomes, a combination of the following circular DNA markers was used: 172 kb EBV episomes co-extracted from the D98/Raji host cells as an internal standard, BAC clones containing anonymous human DNA of 140 kb, 180 kb and 250 kb (kindly provided by Eric Lai, Glaxo-Wellcome, Research Triangle Park, NC, USA), a PAC clone containing 170 kb human DNA from the HPRT region (kindly provided by Pieter de Jong, Rossevelt Park Cancer Center, Buffalo, NY, USA) and a P1 clone with an 80 kb insert from the human p53 region (Shepherd et al. *supra* 1994). The DNAs were isolated from bacteria by alkaline lysis and run uncut in parallel to the episomes purified from the D98/Raji human cell transformants. l/HinDIII DNA was used as a linear marker.

Fluorescence In-Situ Hybridization (FISH). Metaphase and interphase spreads were prepared as recently described (Banerjee et al., *Nature Med.* **1**, 1303 (1995); Sun et al., *Gene Ther.* **3**, 1081 (1996)) from untransfected and BAC-HAEC transfected D98/Raji cells. Slides were probed with the parental BAC vector

pBeloBac11 (Kim et al., *supra*) labelled with a Prime-It-Fluor kit (Stratagene) to specifically detect the pBH140 vector component of the HAEC clones. Samples were visualized at 1000 x magnification on an Olympus IMT-2 microscope equipped with epifluorescence and filters for fluorescein detection. Color pictures were taken using Kodak Ultra Gold film (ASA400). HAEC-specific signals in 50 nuclear/chromosomal spreads were counted per experiment.

RT-PCR. Transcription of the human β-globin gene was analyzed by reverse transcription-PCR (RT-PCR) across its second intron as recently described (Sierakowska et al., *Proc. Natl. Acad. Sci. USA* **93**, 12840 (1996)) . Briefly, total cellular RNA was isolated with TRI Reagent (Molecular Research Center, Cincinnati) and analyzed by RT-PCR (RT-PCR) using rTh DNA polymerase as suggested by the manufacter (Perkin-Elmer). Forward and reverse primers spanning exons 2 and 3, respectively, generated a 231 bp product from the correctly spliced human β-globin RNA. For quantitative analysis, the RT-PCR was carried out with [α-^{32}P]dATP for no more than 18-20 cycles. Under these conditions, the amount of PCR product was proportional to the amount of correctly spliced input RNA (Sierakowska et al., *supra*). No product was detectable without the reverse transcrition reaction. The RT-PCR products were separated on a 7.5% nondenaturing polyacrylamide gel and quantitated by scanning the gel in a phosphoimager (Molecular Dynamics).

B. RESULTS

Retrofitting large PAC/BAC inserts into a hybrid BAC-HAEC episomal vector. As shown in **Fig. 1A**, the pBH140 vector is based on the first generation HAEC system (Sun et al., *supra* 1994) and the BAC vector pBeloBAC11 (Kim et al., *supra*) . In particular, it contains the EBV-specific latent replication origin oriP and its CMV-driven EBNA-1 transactivator allowing episomal maintenance of large circular plasmids in human cells. A schematic flow chart of the cloning strategy is shown in **Fig. 1B**. A PAC clone (Ioannou et al., *supra*) which carried a 185 kb human fragments spanning the entire β-globin locus including its LCR was used. This insert, p148, was removed by NotI digestion, purified by pulsed field agarose gel electrophoresis, ligated into the NotI-linearized dephosphorylated BAC-HAEC vector pBH140 and transformed into bacteria using chloramphenicol selection. After

purification of the β-globin/BAC-HAEC (p148BH) plasmid DNA from bacteria, the size of the human genome insert shuttled into the BAC-HAEC vector was analyzed by NotI digestion and pulse-field gel electrophoresis using BAC and PAC size markers. As shown in **Fig. 1C**, the β-globin human DNA insert released from the pBH140 vector migrated as a 185 kb fragment relative to various linear DNA size markers.

Episomal shuttling of large BAC-HAEC clones from bacteria into human cells. To establish the 185 kb human β-globin insert into human cells as circular extrachromosomal elements, 2.5 ug of p148BH BAC-HAEC DNA was lipofected into EBNA-1 expressing human D98/Raji cells. This EBV-positive EBNA-1 expressing cell line was chosen because it has previously been used to establish large pBR322-based HAECs carrying 80-350 kb of anonymous human genomic inserts (Sun et al., 94). After 2 weeks of hygromycin selection, approx. 50 clones were obtained and pooled. All experiments described below were performed on such pooled clones. Episomal DNA was isolated and loaded on neutral agarose gels using conditions which resolve large circular DNA molecules (Sun et al., *supra* 1994) . As shown in **Fig. 2A** (lane 5 left/panel) as well as in **Fig. 2B** (lane 2 left/panel), a single band of circular p148BH DNA was detected by Southern blot hybridization using a vector-specific hygromycin probe. The size of the p148BH episomal band was estimated in following two different ways (**Fig. 2A**). First, two anonymous BAC-HAECs of 140 and 180 kb respective size (lanes 1-2) as well as the bacterial p148BH DNA (lane 3) were loaded in parallel to the p148BH episomes isolated from D98/Raji cells (lane 5) and the resulting filter was hybridized with the vector-specific hygromycin probe (left/panel). Second, after stripping the hygromycin probe from the filter, it was re-hybridized with the EBV-specific BamH1 "W" probe to detect the position of migration of the endogenous circular 170 kb EBV genome Raji (lane 5 right/panel). As shown in **Fig. 2A**, p148BH episomes isolated from the pool of human cell transformants (lane 5 right/panel) run to approx. the same position as the 180 kb BAC bacterial marker (lane 2 right/panel) and the endogenous 172 kb EBV DNA marker (lane 5 left/panel). Furthermore, the position of migration of these BAC-HAEC b-globin plasmids (lane 5) appeared similar to the original DNA prepared from bacteria

(lane 3). Hence, the transfected p148BH had maintained its large insert size during episomal shuttling and establishment in human D98/Raji cells.

Long-term stability of the large genomic BAC-HAEC clone in human cells: To test the stability of the shuttled BAC-HAEC clones in human cells as a function of time, the pool of D98/Raji cell transformants containing the 185 kb human β-globin plasmids were cultivated continuously for more than one year under hygromycin selection (**Fig. 2B**, lane 2). For comparison, an anonymous 180 kb BAC-HAEC clone also established in D98/Raji cells was grown and analyzed in parallel cultures (**Fig. 2B**, lane 1). First, unrestricted p148BH episomes extracted from the late passage D98/Raji cells were compared to the 180 kb anonymous ones by agarose gel electrophoresis and Southern blot hybridization with the vector-specific hygromycin probe (**Fig. 2B**, left lanes). The human β-globin (lane 2) and anonymous (lane 1) episomes migrated at approx. the same position. Second, a duplicate gel blot was probed with the EBV-specific "W" probe to locate the position of migration of the 170 viral genome (**Fig.2B**, right/panel). Probing with the BamH1 W fragment revealed a single EBV band at the expected 170 kb position in D98/Raji cells transformed with either the anonymous (lane 1) or the β-globin (lane 2) BAC-HAEC plasmids. Although as illustrated in lanes 5 and 6 slight variation in the migrations of a large supercoiled DNA as the EBV genome can be observed between samples, such differences are not reproducible between preparations. Third, while the relative EBV copy number was similar in the p148BH and anonymous BAC-HAEC cell transformants (right/panel), the higher intensity of the p148BH band (lane 2, left and middle panel) compared to that of the anonymous 180 kb clone (lanes 1 left and middle) indicated a higher copy number of the former relative to the later. Such a fluctuation in copy number with various human inserts has also been observed with anonymous human HAEC clones (Sun et al., *supra* 1994). Altogether, these results illustrated i) the stability of such large 180-185 kb BAC-HAEC clones during a one-year cultivation period, and ii) the potential influence of the human genomic insert on the number of BAC-HAECs per cell.

Extrachromosomal maintenance and copy number of the BAC-HAEC in human cells. To determine the copy number of the human β-globin BAC-HAEC episomes and to analyze the potential integration of the plasmids into human

chromosomes, FISH was performed with a vector-specific hybridization probe (Banerjee et al. 1995; Sun et al. 1996) . While little FISH signal was detectable in the parental D98/Raji cell spreads (**Fig. 3A-B and E**), the majority of chromosomal spreads from the p148BH-transformed cells carried 11 to 15 plasmids per nuclei (**Fig. 3C-D and E**), confirming the conclusion of the multicopy status of the β-globin episomes in the D98/Raji human cells (**Fig. 2B**). Interestingly, β-globin HAEC vector-specific signals in the interphase nuclei appeared to be clustered and located in the proximity of the peri-nuclear membrane. As illustrated in **Fig. 3A**, all the episome-specific hybridization signals on metaphase spreads were either located on only one chromatid of a chromosome or at different positions in the case of two or more signals on the same chromatids pair. Since chromosomal integration will be vizualized as a symmetrical double signal ("doublets") located at the same position on both chromatids of a pair (Sun et al., *supra* 1996) , it can be concluded that there was no indication of BAC-HAEC integration into D98/Raji chromosomes.

Persistent genomic transcription on self-replicating large BAC-HAEC plasmids in human cells. To test for potential transcription from the β-globin cluster established as p148BH episomes in human D98/Raji cells, RT-PCR was applied to the β-globin gene transcripts using primers spanning its intron 2 (**Fig. 4**, bottom section) . As expected for such non-erythroid cells, the untransfected D98/Raji cells did not transcribe their endogenous adult β-globin gene (**Fig. 4A**, lane 7). However, RT-PCR on total RNA prepared from the D98/Raji cells transformed with the p148BH episomes generated a unique 231 bp product (**Fig. 4A, lanes 4-5, 8-9**) identical in size to that obtained from primary human blood cells (**Fig. 4A**, lanes 1 and 11). In addition, no other product was detectable (data not shown) indicating that all the primary transcripts made on the β-globin genomic template were correctly spliced, at least across this intron 2 (Sierakowska et al., *supra*) . Interestingly, active transcription of the exogenous human β-globin gene was observed after one year of continuous cell cultivation as shown by the generation of identical RT-PCR products from late passage samples (**Fig. 4B**, lanes 2 and 4). Variation in the number of PCR cycles (**Fig. 4A**, lanes 4 and 5, 8 and 9) or the amount of RNA/DNA templates (**Fig. 4A**, lanes 4 and 8, 5 and 9) indicated proportionality of the final products as reported previously (Sierakowska et al., *supra*). Such a quantitative RT-PCR analysis (**Fig. 4B**)

showed that the amount of transcription from the p148BH-transformed D98/Raji cell pool (**Fig. 4B**, lanes 2 and 4) reached, respectively, 15 to 70% of three different HeLa clones (**Fig, 4B**, lanes 7-12) expressing integrated CMV-driven human β-globin minigenes (Sierakowska et al., *supra*). This observation confirmed the sustained transcription of the extra-chromosomal genomic β-globin gene during prolonged *in vitro* cell cultures. Hence, a persistent transcriptional activity from the large BAC-HAEC clone p148BH occured in parallel to the long-term autonomous replication and episomal stability.

C. DISCUSSION

A novel hybrid BAC-HAEC vector pBH140 has been developed for shuttling large human inserts from pre-existing bacterial BAC or PAC libraries into human cells following standard subcloning techniques. The presence of the human herpesviral replication origin oriP on the BAC-HAEC vector allowed stable extrachromosomal maintenance of 200 kb episomes in EBNA-1 positive human cells for more than one year. Importantly, active and persistent transcription of a human gene on the extrachromosomal large genomic inserts was observed during this long-term cultivation period.

Shuttling and stability of large BACs/PACs into human cells. The universal design of this "second-generation" HAEC vector (**Fig. 1**) should allow the shuttling of any large genomic insert from pre-existing P1-, PAC- or BAC-based libraries into EBNA-1 positive human cells. In addition, the use of the pBH140 system is not restricted to human cells expressing a pre-established EBNA-1 gene (Scott and Vos, unpubl. data), since the BAC-HAEC vector itself carries a constitutively expressed EBNA-1 (**Fig. 1A**). The current cloning capacity of the BAC-based pBH140 vector, up to at least 200 kb, will be sufficient to span most human genes on a single genomic insert. The criteria in this study for the faithful shuttling of a BAC/PAC-based large human genome insert into human cells as BAC/HAEC is based on the relative migration rate of supercoiled DNA as analyzed by agarose gel electrophoresis. This method allows for rapidly estimating and accurately comparing the size of circular DNA in the 80 to 350 kb range extracted either from bacterial or mammalian cells (Sun et al., *supra*; Sun & Vos, In *Methods in*

Molecular Engineering, 167 (K. Adolph, Ed. 1996)). However, slight differences in migration rates of large supercoiled DNA can occur between sample preparations. In addition, this procedure will not necessarily detect small sequence alterations or minor rearrangements. Hence to refine the analysis of the integrity of the shuttled clone, one can perform fine restriction genomic fingerprinting (Sun et al., *supra* 1994; Sun & Vos, *supra*) and, if desirable, large scale sequencing. Obviously, functional testing of genomic activities carried by the inserts such as a putative gene will complement any structural analysis.

A yeast-based system for shuttling up to 660 kb large YACs as oriP-based circular episomes in EBNA-1 positive human cells has been recently described (Simpson et al., *supra*) . Such a system offers a complementary approach to the BAC-HAEC strategy, particularly for shuttling genomic clones larger than 200 kb. A potential drawback of the YAC procedure is its dependence on two subsequent homologous recombination steps performed *in situ, i.e.* circularization of the linear YAC and retrofitting of oriP/hygromycin into the circular YAC. The retrofitted circular YAC clones were introduced into human cells by spheroblast fusion, a gentle but inefficient method of DNA delivery, *i.e.* 1 clone/$5x10^6$ fused cells (Simpson et al., *supra*) , potentially complicated by the co-transfer of yeast chromosomal DNA. Using the BAC-HAEC system, a larger number of clones, i.e. 50 clones/10^6 transfected cells, can be obtained by lipofection as a DNA transfer technique allowing to rapidly generate large numbers of cell transformants. Similarly to YAC technology, the large genomic inserts on BACs can be specifically modified *in situ* using genetic-based homologous targeting techniques (Xu et al., *Nature Biotech.* **15**, 859 (1997)) . BAC-based clones in bacteria are easier to handle and appear stable (Kim et al., *supra*) . In addition, the BAC-HAEC system may also be used for subcloning the genomic region of interest from large YAC inserts by partial restriction into the pBH140 vector.

Episomal persistence and copy number in human nuclei. As reported with pBR322- or YAC-based large oriP plasmids in human cells (Sun et al., *supra* 1994; Simpson et al., *supra*) , there was no indication of BAC-HAEC integration into human chromosomes by FISH analysis on metaphase spreads (**Fig. 3C**). Although some variation in the episome copy number among individual nuclei occured, an

average of 11 - 15 signals per nucleus was observed (**Fig. 3E**). As such, p148BH was established at a relatively high copy number compared to previous studies (Banerjee et al., *supra*; Simpson et al., *supra*; Sun et al., *supra* 1996) . Such a difference could be due to the respective DNA delivery methods, i.e. DNA transfection (Sun et al., *supra* 1994) , viral infection (Banerjee et al., *supra*; Sun et al., *supra* 1996) and yeast spheroplast fusion (Simpson et al., *supra*) . Alternatively, the presence of a second origin of replication , i.e. the insert-based origin located upstream from the β-globin gene (Aladjem et al., *Science* **270**, 815 (1995)) potentially active on the same p148BH episome may enhance the copy number per nuclei. In future studies, it will be interesting to test whether the globin origin can replace oriP, especially in human cell types where oriP episomes cannot replicate effectively (Vidal et al., *Biochim. Biophys. Acta* **1048** , 171 (1990)) or in mammalian cell species where oriP replication is non-permissive (Yates et al., *supra*) . For example, oriP/EBNA-1 vectors cannot persist episomally in rodent cells. However, recent studies indicate that 100 kb anonymous human inserts carrying oriP can replicate autonomously in murine cells expressing EBNA-1 (Kelleher et al., *Nature Biotech.* **16**, 762 (1998)). It has been speculated that such Mouse Artificial Episomal Chromosomes (MAECs) carry active replication origins within the large human genomic insert (Vos, *Curr. Opin Genet. Dev.* **8**, 351 (1998)). By analogy, the replication origin from the human β-globin locus may also complement an inactive oriP in rodent cells.

Sustained genomic activation of BAC-HAECs in human cells. This study revealed transcription of the human β-globin from 200 kb autonomously replicating episomes in stable human cell transformants. Specifically, correctly spliced β-globin mRNA identical to that from control human red blood cells was detected in D98/Raji cells containing p148BH but not in untransfected D98/Raji cells. In addition, quantitative RT-PCR analysis indicated i) a level of transcription nearly as high as that of CMV-driven β-globin mini-genes integrated in HeLa cell chromosomes (Sierakowska et al., *supra*) , and ii) importantly, the stable persistence of such unselected transcription for over a year in tissue culture. We have not been able to detect β-globin protein using an immunoblot assay on D98/Raji carrying the p148BH episomes, a result most likely due to the significant lower sensitivity of this assay than RT-PCR (Sierakowska & Kole, unpubl. observ.). However, the RT-PCR product is a

reliable measure of the level of β-globin gene expression since in HeLa cells it correlated well with the level of translated β-globin protein (Sierakowska et al., *supra*). In adults, transcription from the β-globin cluster on chromosome 11 occurs in erythroblasts. Activation of globin gene expression has been demonstrated in transient heterokaryons between HeLa cells and mouse erythroleukemia (MEL) cells (Baron and Maniatis, *Cell* 4, 591 (1986)), in MEL cells after introduction of a YAC-based β-globin cluster (Peterson, Zitnik et al., *supra*) and in transgenic mice carrying the corresponding human β-globin YAC (Peterson, Clegg et al., *supra*) . Since all cases involved an erythroid background, the observation of β-globin expression in an non-erythroid human cell line is intriguing. Two models may explain this observation based respectively on general or specific promoter activation. On the one hand, transfection of large genomic fragments as nacked DNA in somatic cells may allow basal transcription of a tissue-specific gene in the absence of its tissue-specific transcription factors. Such transient ectopic promoter activation would occur prior to nucleosomal repression (Wolffe, *J. Cell. Sci.* **99**, 201 (1991)) and would establish a transcriptionally active domain (Dillon and Grosveld, *supra*). By avoiding chromosomal integration and associated transcriptional shut-off (Milot et al, *Trends Genet.* **12**, 123 (1996)), extrachromosomal replication would help preserve stable inheritance of such basal transcription in dividing cells (Wolffe, *in DNA Replication in Eukaryotic Cells,* 271 (M. DePamphilis, ed. 1996)). On the other hand, whole cell fusion between the epithelial carcinoma HeLa-derived D98 line with the B lymphoma Raji line may have inappropriatly activated erythroid-specific factors involved in β-globin transcription. Tissue-specific β-globin expression is dependent on the seeding of an active chromatin configuration as well as the presence of the LCR (Peterson et al., supra) . The human β-globin DNA insert on p148 does include and extend beyond the LCR (P. Ioannou, pers.com.). Although the D98/Raji line does not transcribe its endogenous β-globin genes (**Fig. 4**), transcriptional reprogramming of these chromosomal genes may not occur due to dominant chromatin repression. In contrast, the transfected nicked DNA may be accessible to such "ectopically-activated" tissue-specific factors. Clearly, the mechanism and potential generality of this phenomenon will have to be further analyzed, particularly *in vivo* using animal models of human syndromes.

Current OriP/EBNA-1 plasmids require selection because of low transfection efficiency and plasmid loss. The 100-200 kb oriP-based HAECs persist with a half-life of approx. 3 months in actively dividing human cells grown in the absence of hygromycin selection (Banerjee et al., *supra*; Gulino & Vos, unpubl. observ.). Considering that most cells in the human body are non-dividing or semi-quiescent, such an efficiency of persistence may be sufficient, or may require medium-term repetitive treatment protocoles. If so, *ex vivo* gene therapy strategies relying on *in vitro* selection of stably transduced cells could be based on a dominant selectable marker such as hygromycin. Alternatively, one could use *in vivo*-selectable resistant markers such as the methotrexate/DHFR system (Licht et al., *Stem Cells* 15, 104 (1997)). Hence, a gene therapy based on episomal genomic activation may become an attractive alternative strategy for treating those diseases recalcitrant to long-term phenotypic correction via gene expression.

EXAMPLE 2

An Enhanced EBNA1 Variant with Partial IR3 Domain for Long-Term Episomal Maintenance and Transgene Expression of OriP-Based Plasmids in Human Cells

A successful extrachromosomal, or episome-based, somatic gene therapy system for inherited genetic disorders has to fulfill at least three basic requirements. First, the vector would have to persist for prolonged periods in the nucleus of the cell without integrating into the host's genome. Ideally, a single or multicopy plasmid whose replication could be efficiently regulated to only once per cell cycle should be utilized. Integration of a therapeutic episome into the host's chromatin is undesirable for several main reasons: integration could inactivate any gene residing on the vector; integration has a distinct possibility of activating or inactivating important genetic loci potentially resulting in deleterious consequences. Second, the newly replicated plasmid(s) would have to segregate to daughter cell nuclei efficiently, ensuring a steady state copy number of the plasmid. Efficient episomal maintenance (replication and segregation) would ensure that the therapeutic vector persists in the treated cells for the entire life of the individual. Finally, the vector would deliver sustained expression of the therapeutic gene for prolonged periods, avoiding repressive mechanisms of gene regulation such as chromatin condensation and methylation at

CpG sites in promoters.

The Epstein Barr Virus (EBV) presents itself as a useful viral system to potentially meet the above criteria as an episomal gene delivery vector. EBV is a large 172 kb double stranded DNA herpesvirus that preferentially infects B lymphocytes, although it has been detected in a variety of other tissues as well (Busson et al, *Proc. Natl. Acad. Sci. USA.* **84**, 6262 (1987); Labrecque et al., *Cancer Res.* **55**, 39 (1995); Harn et al., *J. Clin. Gastroentero.* **20**, 253 (1995)). Once the linear viral DNA enters the cell it recircularizes and subsequently establishes a latent pattern of replication in the nucleus of the infected cell for the life of the individual. The viral genome is then replicated only once per cell division cycle (Hampar et al. *Proc. Natl. Acad. Sci. USA.* **71**, 631 (1974); Adam, *J. Virol* **61**, 1743 (1987); Yates and Guan. *J. Virol.* **65**, 483 (1991)) by the host's DNA replication machinery in synchrony with the host's genome. The large viral episome has been found to maintain a stable copy number of between 5 and 50 per cell nucleus in Burkitt's lymphoma (BL) and lymphoblastoid cell lines (LCL).

Small helper-dependent miniEBV plasmids have been developed which carry the latent origin of replication of EBV (oriP) and other *cis*-DNA elements required for producing an infectious amplicon virus capable of establishing latency in infected cells (for review see Vos, in *Viruses in human gene therapy,* 109 (J.-MH. Vos Ed., Carolina Academic Press & Chapman & Hall: Durham N.C., USA & London, UK, 1995)) . Derivatives of this miniEBV have been successfully used to transport and express several recombinant genes in B lymphocytes (Banerjee et al., *supra* (1995); Wang et al. *Hum. Gene Ther.* **6**, 1005 (1995); Sun et al., *supra* 1996)

Latent replication of the EBV genome depends on only one viral encoded DNA binding protein referred to as Epstein Barr nuclear antigen 1 (EBNA1). This antigen has no detectable intrinsic enzymatic activity but binds to multiple locations in the EBV genome, specifically at the origin of bidirectional replication (OBR, or dyad symmetry element, DS) and at a cluster of 20 consecutive binding sites called the family of repeats (FR). These two *cis* elements, together referred to as oriP, are separated by approximately 1000 bp of nonessential DNA and function in latent DNA replication, plasmid maintenance, and transcriptional enhancement, all of which

depend upon the binding of EBNA1 protein (Yates, *supra* 1996). Stable maintenance of oriP-containing plasmids in dividing cells involves two EBNA1-dependent functions: the initiation of plasmid replication at the OBR (Gahn and Schildkraut, *Cell* **58**, 527 (1989); Wysokenski and Yates, *J. Virol.* **63**, 2657 (1989); Harrison et al., *J. Virol.* **68**, 1913 (1994)) and a replication independent plasmid maintenance function. EBNA1 dimers bound to oriP physically link FR to the OBR (DS), resulting in the formation of a loop consisting of the intervening DNA separating these two elements (Frappier and O'Donnell, *Proc. Natl. Acad. Sci. USA.* **88**, 10875 (1991); Su et al., *Proc. Natl. Acad. Sci. USA.* **88**, 10870 (1991); Middelton and Sugden, *J. Virol.* **66**, 489 (1992)). This linking activity is required for the three activities of oriP mentioned above (Yates, *supra* 1996)

The EBNA1 protein has been extensively studied through detailed mutational analysis and several functional domains of EBNA1 have been mapped (**Figure 5**) (Yates and Camiolo, *Cancer Cells* **6**, 197 (1998); Ambinder et al., *J. Virol.* **65**, 1466 (1991); Inoue et al. *Virology* **182**, 84 (1991); Chen et al., *J. Virol.* **67**, 4875 (1993); Goldsmith et al., *J. Virol.* **67**, 3418 (1993); Mackey et al., *J. Virol.* **69**, 6199 (1995)) Although the nuclear localization signal (NLS), the DNA binding and dimerization domain, and the DNA linking (or looping) domains have been mapped, a consensus transcriptional activation domain has not yet been discovered. The amino terminal one-third of EBNA1 contains a large, variable repeat region with a high GlycylAlanine content, referred to either as internal repeat 3 (IR3) or GlyAla domain (Yates et al., *Nature* **313**, 812 (1985)). Although it has been reported that deletion of a large segment of this domain has no profound affect on the function of EBNA1 in transient experiments (Yates and Camiolo, *supra* 1998; Yates et al., *supra* 1985), it has been shown to interfere with antigen processing and/or presentation of EBNA1 to the cellular immune compartment (Klein *Cell* **77**, 791 (1994); Levitskaya et al., Nature **375**, 685 (1995)) Interestingly, it was also shown that this immune escape function can be transmitted to other proteins when fused to this domain (Levitskaya et al., *supra*).

Several lines of evidence indicate a potential effect of the GlyAla repeat on EBNA1 function. Short-term DNA transfection experiments comparing mutant and wild-type EBNA1 proteins on reporter constructs revealed a subtle effect on episomal

replication, reporter gene transactivation, and plasmid copy number (Yates and Camiolo, *supra*). In long-term cell transformation experiments, there has been observed a differential stability of transduced the pH210 vector (**Fig. 6**) in different EBV positive human B cell lines latently infected with different strains of EBV. Specifically, it was observed that the B lymphoma Raji cell line was able to maintain oriP-based reporter expression more efficiently than the B lymphoblastoid cell line HSC536. Bannerjee et al., *supra*) Recent experiments with stably transduced pH210 vector in additional EBV-positive Burkitt's lymphoma lines further support a correlation between the isoform of EBNA1 being expressed and long-term stability of OriP-based plasmids. Although such an effect could be due to genetic differences between these cell lines, it could also be a function of the EBNA1s being expressed by different strains of EBV resident in these cells. As the molecular weight of EBNA1 can vary due to length variations in the GlyAla domain, there was interest in determining whether this unusual structure may contribute to stability of OriP/EBNA1 episomes. HSC536 cells harbor the B95.8 strain of EBV and express an EBNA1 of approximately 76 kDa, with approximately 268 amino acids composing the GlyAla domain. The Raji strain of EBV expresses an EBNA1 with a molecular weight of 67 kDa due to a reduction in the GlyAla domain to approximately 170 amino acids (see below).

To investigate this further the EBNA1 gene was isolated from the resident EBV in the non-producer African Burkitt's lymphoma Raji using a novel cloning strategy. The result of a major truncation (224 amino acids) of this domain on EBNA1 activity was also examined. Here it is show in long-term gene transduction experiments that the GlyAla repeat encoded in the EBNA1 protein positively influences long-term maintenance of oriP-based plasmids in human B cells. These results could have important implications for the design and development of episomal gene deliver vectors which rely on efficient and optimal EBNA1 function to maintain themselves as episomal vectors in the host's cell nucleus.

A. MATERIALS AND METHODS.

Cells and cell culture. Epstein Barr virus (EBV) positive Raji and HH514 cells (Banerjee. *supra*; Sun, *supra* 1994) and EBV negative DG75 and BJAB cells (a gift from Dr. Shannon Kenney, UNC, Chapel Hill, NC) were grown in RPMI 1640

medium supplemented with 10% heat inactivated fetal bovine serum, glutamine, and antibiotics.

Subcloning of Raji EBNA1. Large covalently closed EBV episomes were prepared from Raji as previously described (Banerjee, *supra*; Sternas et al., J. Virol. 64(5), 2407 (1990)). Approximately 20 ug nucleic acid were digested with the restriction enzymes BamHI and HindIII for 2 hours at 37°C until completion. Double digested restriction fragments were shotgun ligated into the plasmid pGEM3zf(+) which had been previously digested with the same enzymes. Ligation products were transformed into competent XL1 blue MRF' bacteria and plated on LB agar plates containing ampicillin, and X-gal. Approximately 500 white colonies were picked and transferred to large 150 mm LB-AX plates and screened by colony hybridization using the EBNA1 gene as a probe. Seven colonies were positively identified and their partial EBNA-1 sequence was shown to be identical. The complete nucleotide sequence of one randomly chosen Raji EBNA1 gene was determined by manual sequencing.

EBNA1 expression constructs. The plasmids pH210 and pH211 (**Fig. 6**) with no EBNA-1 contain the following *cis*-acting elements: i) the lytic origin of replication of EBV (oriLyt), ii) packaging signals (terminal repeat, TR), and iii) the latent origin of plasmid replication and EBNA1 binding sites (oriP). A HindIII restriction fragment containing the CMVEBNA1 (B95.8 derived EBNA1) expression cassette from the commercially available pCMVEBNA1 plasmid (Invitrogen) was inserted into the HindIII site of the miniEBV plasmid pH211 to give CMVB958.E1 (**Fig. 6**). To construct the Raji and the IR3del EBNA1 expression cassettes, the AvrII to SacII restriction fragment of the EBNA1 coding region in pCMVEBNA1 was replaced or swapped by the analogous restriction fragment from the Raji and IR3del EBNA1s. The IR3del EBNA1 coding region was derived from the plasmid pREP7 from Invitrogen. This clone of EBNA1 has a large deletion in the GlyAla repeat region. The HindIII expression cassette from these subclones was then transferred to the singular HindIII site of plasmid pH211 to give CMVRaji.E1 and CMVIR3del.E1, respectively (**Fig. 6**). All clones were analyzed by restriction analysis to confirm and to determine orientation of inserts.

Transfection of mini-EBV plasmids. 10 ug of the plasmid pH211 and

EBNA1-expressing derivatives (**Fig. 6**) were electroporated into $1x10^7$ EBV negative DG75 cells in accordance with known techniques. Stable cell pools were selected by growth in the antibiotic Hygromycin B at 200 ug/ml. The positive control for episomal replication was provided by Raji cells stably transformed with pH210. *Supra.*

Western blot procedure. Approximately $1x10^7$ cells were harvested, washed in PBS, and resuspended in 300-500 ul ELB (250 mM NaCl, 50 mM Hepes, pH7.0, 5 mM EDTA) containing 1.0 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM dithiothreitol (DTT), 0.1% NP-40. Protein concentration was determined using Coomassie Plus reagent. For each experiment 40 ug protein was loaded per lane and separated on 7.5 % SDS-PAGE. Rainbow molecular weight markers (Amersham) were used in each gel. Proteins were transferred to nitrocellulose (Micron Separations) in a Semidry Transfer unit (Biorad) for 30 minutes at 20 volts. The membrane was blocked overnight at 4^oC in TBST containing 3% nonfat dry milk. The membrane was then incubated for 30 minutes at room temperature with an EBNA1 specific monoclonal antibody, EBNA1.OT1x (gift from J.M. Middeldorp & S.D. Hayward, John Hopkins School of Medicine, Baltimore, MA, USA)(Chen, *supra* 1993). The secondary antibody was goat anti-mouse IgG. Proteins were detected by ECL (Amersham).

Episome purification procedure and Southern blot analysis. The preparation of the episomes for analysis were performed in accordance with known techniques. Briefly, $1x10^7$ cells were harvested and washed in PBS. Cells were lysed at pH 12.45 in 2 ml cell lysis buffer with vortexing. Lysates were incubated at 30^oC before the step-wise addition of 400 ul 1M TrisCl pH 7.0, 264 ul 5M NaCl, 12 ul 10 mg/ml proteinase K. The lysates were then incubated for at least 2 hours at 37^oC. Samples were organic extracted and ethanol precipitated. The O.D. at 260 nm was determined to calculate the concentration of total nucleic acid. Equivalent amounts of nucleic acid were (30 or 40 ug) digested with Bgl II restriction enzyme to linearize the plasmid and separated on 0.8% agarose pulse field gels. Nucleic acids were then transferred to nylon membranes (Micron Separations) in 0.2 N NaOH overnight. Blots

were then analyzed with probes synthesized using Primagene (Promega). Signals on the blots were then quantified using Molecular Dynamics Phosphorimager. The human mitochondrial probe was derived from the PCR amplification of a small region within the 5.4 kb HindIII fragment of human mitochondrial DNA (16 kb). The sequence of the primers that were used are as follows:

5'TTCTCCTTACACCTAGCAGG (SEQ ID: 1),

5'TAGCGATGATTATGGTAGCG (SEQ ID: 2).

The annealing temperature of the primer combination was 58°C.

Colorimetric ONPG assay (lacZ assay). Approximately $2x10^6$ cells were harvested and washed in PBS. Cells were resuspended in 0.5 ml PBS and 0.5 ml 2x ONPG (O-nitrophenyl-b-D-galactopyranoside) containing 0.1% NP40 was added and samples were vortexed to lyse the cells (Lim and Chae, *BioTechniques* 7, 576 (1989). Samples were then incubated for at least 2 hours at 37°C. To quantify the amount of lacZ expression from each of the transfected pools the lysates were diluted at least 2-fold and the O.D. was determined for each sample at 420 nm. Control lacZ expressing cells were Raji cells that had been stably infected with miniEBV pH210. This polyclonal cell line was created by infecting Raji Burkitt's lymphoma cells with pH210 miniEBV as previously described (Banerjee, *supra*).

B. RESULTS

1. Cloning and Functionality of various EBNA1 isoforms

Cloning of a novel EBNA1 from the EBV-positive non-producer strain Raji. Because Raji EBV cannot be produced from its latently infected host cell, an alternative cloning strategy was derived based on selective alkaline extraction and purification of the latent 172 kb circular viral episome. High molecular weight extrachromosomal DNA was extracted from Raji by an alkaline lysis procedure as described above. As described above, the lysate containing large circular EBV episomes was double digested and shotgun ligated into vector pGEM3zf(+) (Promega). Approximately 500 transformants were screened by colony hybridization and seven positive clones were identified. Miniprep DNA (Flexiprep, Pharmacia) was

isolated from these seven clones and sequencing (Sequenase, US Biochemical) of the first 200 b of unique sequence directly 3' to the internal repeat (IR3) domain confirmed that all EBNA1 clones were identical. The complete DNA sequence of one clone was determined (with the exception of the GlyAla domain) and compared to the standard B95.8 derived EBNA1 sequence. A comparison of the amino acid differences between several EBNA1 proteins is summarized in **Figure 5**. As previously shown (Hennessy et al., *Science* **220**, 1396 (1983)) it was determined by restriction analysis that the GlyAla repeat (IR3) of the Raji EBNA1 gene was approximately 300 bp shorter than that of the B95.8 (which is approx. 807 bp) EBNA1 gene. This is in accordance with the size difference between the two EBNA1 proteins (76 kD vs. 67 kD).

Construction of oriP plasmids expressing different EBNA1 isoforms. In order to test the expression and function of each EBNA1 protein a series of oriP vectors were created using the same expression cassette which encode different EBNA1 genes. To test the affect of the GlyAla domain on EBNA1 function the coding region of a truncated form of the B95.8 EBNA1 derived from plasmid pREP7 (Invitrogen) was included. The EBNA1 protein expressed from this gene is approximately 46 kDa since it encodes only a small portion of the original GlyAla domain (44 amino acids from an original 268). The backbone of the plasmids used in these analyses is based on the parental vector pH211 depicted in **Figure 1**. This oriP-based plasmid also encodes an SV40 promoter-driven lacZ gene for assaying transgene expression. HindIII expression cassettes, derived from the plasmid pCMVEBNA1 (Invitrogen), were engineered encoding different isoforms of EBNA1 transcribed from the viral cytomegalovirus (CMV) immediate early promoter (**Figure 6**) and inserted into the HindIII site of pH211. The coding regions of the Raji and IR3del EBNA1 genes were swapped with the EBNA1 coding region of pCMVEBNA1 to create identical expression cassettes for these different isoforms.

Stable Episomal transformation of human B cells with oriP plasmids encoding various EBNA1 isoforms. To determine the effect of GlyAla repeat length on different EBNA1 isoforms each oriP/EBNA1 expression construct was transfected into DG75 cells by electroporation. This EBV negative Burkitt's lymphoma cell line was chosen because of its lack of endogenous EBNA1 (Ben-Bassat et al., *Int. J.*

Cancer 19, 27 (1997)). To minimize the effect of clonal differences in gene expression, hygromycin resistant cell pools were used throughout this study. Episomes were prepared from stably transformed pools at 5 weeks post-transfection and analyzed by Southern blotting to determine the extrachromosomal status of the various plasmids. To enhance signal detection episomes were linearized with the single cutter Bgl II and resolved by agarose pulse field gel electrophoresis (PFGE). Southern analysis revealed that full-size, unrearranged 23 kb episomes were present in cells transfected with all three plasmids (**Figure 7A,** lanes 1,2,3). The BglII linearized plasmid pCMVB95.8.E1 was included in lane 4 as a molecular weight control. Thus, all three isoforms of EBNA1 appeared to support extrachromosomal plasmid maintenance.

Relative expression and sizes of different EBNA1 isoforms. The comparative expression levels and molecular weight of the EBNA1 isoforms were analyzed in the DG75 transformants by Western analysis using an EBNA1 specific monoclonal antibody (Chen, *supra* 1993). Total protein was prepared and equal amounts were separated by SDS-polyacrylamide gel electrophoresis. As illustrated in **Figure 7B** (lanes 3, 4 and 5) the differences in molecular weight of the recombinant EBNA1 proteins predicted from the length variation of the GlyAla repeat were observed. The apparent molecular weight of the B95.8 derived EBNA1 is 76 kDa, the Raji EBNA1 is 62 kDa, and the IR3del EBNA1 is approximately 46 kDa. The recombinant B95.8 EBNA1 expressed in lane 3 appears to migrate identically with the endogenous EBNA1 expressed from the latent viral genome in the B95.8 cell line (lane 1). However, the recombinant Raji EBNA1 in lane 4 migrated slightly faster than the endogenous viral Raji EBNA1 in lane 2. This may reflect differences in post-translational modifications of the EBNA1 proteins in the DG75 and Raji cells or a slight reduction in the size of the GlyAla repeats in the recombinant Raji EBNA1 gene. Densitometry of lighter exposures of this autoradiogram (right panel) revealed that recombinant B95.8 and IR3del EBNA1 proteins were expressed at approximately two thirds and one tenth, respectively, the level of the recombinant Raji EBNA1.

Expression of a transgene from stably transduced oriP episomes. Because sustained production of a transgene is the primary goal in gene delivery vectors, an ONPG colorimetric assay was used to determine the expression levels of the reporter

ß-galactosidase (lacZ) gene encoded on these plasmids (**Figure 6**). As shown in **Figure 7C**, all three episomal plasmids expressing the various recombinant EBNA1 proteins were competent to express high levels of the lacZ gene compared to the amplicon pH210 vector in the Raji positive control. The pH211 control vector with no EBNA1 gene produced little or no detectable lacZ activity, presumably a result of vector integration. These results indicate that cells with stably transduced extrachromosomal plasmids were capable of expressing high levels of reporter gene, while those with presumably integrated pH211 vectors did not as assayed on pooled transformants. Identical transformation experiments (not shown) using EBNA1 expression cassettes driven from a weaker murine cellular PGK promoter were also unable to produce detectable lacZ activity. Even though episomes were no longer detectable in the transformants, the Hyg^r gene must still be actively expressed since the cells remain sensitive to the selection medium. These experiments reveal that regardless of the size of the GlyAla repeat, episomal status can be achieved with subsequent high level transgenic expression.

2. Differential long-term functionality of EBNA1 isoforms.

Long-term *trans*-gene expression from oriP episomes encoding different EBNA1 isoforms. An effective extrachromosomal gene therapy system requires that the vector be stably replicated and efficiently segregated to daughter cells for prolonged periods. To compare plasmid maintenance of the different oriP/EBNA1 containing vectors the transformed pools were grown continuously in the presence of the selection agent (hygromycin) for up to half a year. **Figure 8A** shows a Southern analysis of episomes prepared from these cultures at 5 months post-transfection. The B95.8 and the Raji EBNA1 expressing episomes were easily detectable at equivalent levels (lanes 1 and 2). However, there was complete loss of episomes expressing the truncated EBNA1 protein, CMVIR3del.E1 (lane 3). These results imply that truncated EBNA1 proteins are less efficient in long-term maintenance of oriP/EBNA1 plasmids than wild-type B95.8 or Raji EBNA1 isoforms. Since the only difference between the B95.8 EBNA1 and the IR3del EBNA1 is the size of the GlyAla repeat, this region must influence the stability of OriP/EBNA1 plasmids.

The EBNA1-specific Western analysis of the transformants revealed that

EBNA1 protein expression was dramatically and differentially reduced in both B95.8.E1 and IR3del.E1 clones compared to Raji.E1 in 5 month cultures (**Figure 8B**, lanes 6 & 8). For quantitative comparison, EBNA1 protein expressed in each transformant at one month was included in the analysis (lanes 1-3, **Figure 8B**). Specifically, we were unable to detect EBNA1 protein expressed from the CMVIR3del.E1 construct (compare lane 8 with lane 3). Therefore, the lack of detectable EBNA1 protein in this transformant at 5 months (lane 8) correlated with their lack of episomes (**Figure 8A**, lane 3). A significant reduction in the level of EBNA1 protein expressed from the CMVB95.8.E1 construct was also detected relative to the CMVRaji.E1 transformants (compare lanes 6 & 7 to lanes 1 & 2, respectively). The level of B95.8 EBNA1 protein expressed at 5 months in lane 6 was estimated to be approximately 10 to 15 percent of the Raji EBNA1 in lane 7, indicating a 4 to 6-fold relative reduction in expression compared to the one month time point. Even though there appears to be an approximate 10-fold difference in the steady state level of EBNA1 protein expressed between the CMVRaji.E1 and CMVB95.8.E1 transformants, there is less than a 2-fold difference in plasmid copy number (see **Figure 3A**, compare lanes 1 and 2, see below).

The activity of the lacZ gene expressed from each these constructs was assayed periodically over the course of 6 months. Results from the graphical analysis in **Figure 3C** indicate differential loss of lacZ activity which appears relatively consistent with episome copy numbers and the presence of EBNA1. After 5 months the CMVB95.8.E1 and CMVRaji.E1 clones produced, respectively, 45 and 89 percent of the Raji pH210 control, while lacZ expression from CMVIR3del.E1 was almost undetectable (less than 3 percent of control). In an effort to corroborate the above findings two additional cell lines were transfected with the same oriP/EBNA1 constructs (CMVRaji.E1 and CMVIR3del.E1) prepared at different times. The cell lines were BJAB, an EBV negative Burkitt's lymphoma cell line, and HH514, an EBV positive lymphoblastoid cell line. After transfection (10 ug DNA) stable cell pools were obtained by selection in antibiotic as above and the cells were then cultured for a total of 14 weeks (3.5 months). ONPG assays (**Figure 8D**) on the BJAB and HH514 transformants revealed that lacZ activity from the two CMVEBNA1 constructs differentially declined from initial equivalent levels. After 3.5 months the lacZ

activity determined for the GlyAla deletion mutant in BJAB and HH514 cells was only 20 and 2.9 percent, respectively, the level of the level determined in the CMVRaji.E1 transformants. These results mirror and thus support the data seen with the DG75 transformants.

Differential kinetics of episomal persistence. To improve quantification of the extrachromosomal persistence of episomes as a function of time an internal control was included in the long-term experiments. Since the amount of mitochondrial DNA in a cell line is expected to be stable over time in culture it can provide an adequate extrachromosomal internal control for measuring copy number of co-extracted recombinant episomes. The relative amounts of OriP/EBNA1 plasmids was normalized to the amount of co-extracted mitochondrial genomes. To estimate the copy number per cell of the extracted episomes 100 picograms of HindIII digested pCMVB95.8.E1 DNA was also included in the analysis. **Figure 9A** shows a typical Southern analysis illustrating this normalization assay. Episomes prepared at one month intervals over a half year were linearized and resolved by PFGE as above. The blot was co-hybridized with vector and human mitochondrial (huMit) specific probes. The lower band represents a 5.0 kb huMit DNA restriction fragment (DG75 cells, lane 20), while the top band represents the signal derived from the 20 kb HindIII restriction fragment of pH211 (plasmid copy number standard, lane 21). The faint band just below the vector fragment is nonspecific signal arising from the huMit probe, as seen in the DG75 negative control (lane 20). Lane 1 confirmed the absence of episomal plasmid in DG75 cells transformed by the parental pH211 without EBNA-1. Each triplicate set of lanes represents episomes extracted from CMVB95.8.E1, CMVRaji.E1, CMVIR3del.E1 transformants, respectively, at one month time intervals up to 6 months.

Graphical analysis of episome copy number determination over the 6 month interval (**Figure 9B**) revealed a kinetic difference between the wild-type EBNA1s and the mutant protein. Surprisingly, the results of this experiment showed that the initial copy number of the CMVB95.8.E1 plasmid was approximately 2.5 per cell in the first month post-transfection (lane 2, **Figure 9A**), but increased more than 5-fold by the third month to 16 episomes per cell (lane 8). Subsequently, the copy number began to decline such that by month 6 the episomes were undetectable in lane 17. The initial

copy number of the CMVRaji.E1 expression construct at one month was almost 20 per cell (lane 3), and increased to its maximum value of approximately 24 per cell in the second month (lane 6). Thereafter the copy number began to decline such that by month 6 it was less than 10 percent of its maximum value (approximately 2 per cell, lane 18). The CMVIR3del.E1 expression plasmid only reached a maximum copy number of 4 per cell in the first month (lane 4), was stable until month 3 and became undetectable by month 4 (lane 13). Because the underlying difference between the B95.8 and the IR3del EBNA1 clone is length of the GlyAla repeat, the results demonstrate that this unusual structure in the EBNA1 protein influences long-term stability of oriP-based plasmids in stable B cell transformants.

C. DISCUSSION

This example reveals a novel influence of the GlyAla repeat (IR3) of EBNA1 which affects oriP/EBNA1 plasmid stability and ensuing transgene expression. It was shown that the absence of this domain reduced plasmid persistence in long-term assays of stably transformed human B lymphoid cells. Different isoforms of the EBNA1 protein containing differing amounts of this structure were examined for their ability to maintain OriP/EBNA1 plasmids for up to half a year in tissue culture. The EBNA1 protein of the B95.8 strain of EBV was examined, as well as a GlyAla domain deletion mutant originally derived from the B95.8 EBNA1 gene (Mackey et al., *supra*). The GlyAla domain of this EBNA1 is reduced by approximately 224 amino acids (from an original 268 amino acids). A novel, functional EBNA1 from the non-producer African Burkitt's lymphoma Raji, was cloned and included in the examination. In addition to a total of 15 single amino acid differences in the EBNA1 coding region, the Raji EBNA1 gene encodes a GlyAla domain that is approximately 170 amino acids in length, or 100 amino acids shorter than the B95.8 EBNA1 protein. A series of EBNA1 expression cassettes driven by the strong CMV promoter was constructed. To avoid endogenous EBNA1 activity EBV negative B cells were used in these experiments and pooled stable transformants were used to eliminate any clonal differences in gene expression.

It has previously been observed that pH210-based transgene expression

persisted at a higher level in transduced EBV positive Raji Burkitt's lymphoma cells than in EBV positive HSC536 lymphoblastoid cells (Banerjee et al., *supra*). Over the course of 3 months without selection, it was determined that there was a differential rate of loss in episome copy number of 75 percent and 33 percent in Raji and HSC536 cells, respectively (unpublished data). Plasmid retention depends on the efficiency of replication and segregation of plasmids to daughter cells. The difference in plasmid stability between these two cell lines may be a reflection of inherent metabolic differences between the cancer B cell line Raji and the in vitro transformed lymphoblastoid line HSC536 derived from a Fanconi's anemia patient (Banerjee et al., *supra*). Alternatively, since Raji and HSC536 cells express different isoforms of EBNA1 protein, this could reflect differences in their ability to interact with the OriP element. Yates and Camiolo (*supra*) have observed in short-term transfection experiments that the GlyAla deletion mutant DL7 (identical to the IR3del clone used in these experiments), was able to modestly stimulate (2-fold) replication of oriP-based plasmids, enhance transcription (2-fold) of FR-containing reporter constructs, and allow a higher plasmid copy number (2-fold) per cell as compared to the wild-type control B95.8 EBNA1. These observations suggested a subtle transient effect that the GlyAla domain may impart to the activity of the EBNA1 protein. In contrast, it was observed that the lack of the GlyAla domain negatively influenced EBNA1 function, i.e. promoted plasmid loss. In these long-term experiments (up to 6 months post-transformation), a 10 to 100 fold reduction in episomal plasmid and beta-gal activity was observed over time. Several reasons may explain these different results. First, Yates and Camiolo used the human osteosarcoma 143 line, while this study relies on human B cell lines, the natural site of residence of the latent EBV. The activity of OriP/EBNA-1 system may be differentially affected by tissue-specific factors. Second, the negative effect of the IR3-deleted EBNA-1 only became apparent after at least 3 months of cell culture (Fig. 8C & 9B). Since the previous experiments (Yates and Camiolo, *supra*) did not extend beyond approx. 1 month, a small reduction in a specific EBNA-1 activity may only be manifested through a long-term experiment. Lastly, transcriptional activation and episomal replication by EBNA-1 on OriP may be stimulated by the IR3 deletion, while episomal segregation crucial for long-term maintenance may be selectively inhibited. If so, the IR3-deleted derivative would be a segregation-specific mutant.

Differential long-term functionality of various trans-activating EBNA1 isoforms. The long-term plasmid maintenance assay of **Figure 9A** revealed striking differences in the functional activity of different isoforms of EBNA1. The graphical analysis of these results shown in **Figure 9B** implies differential kinetics for each EBNA1 isoform. Specifically, the relative copy number of the Raji, B95.8 and GlyAla mutant (CMVIR3del.E1) EBNA1-expressing plasmids reached different maximum levels at different time intervals after transfection and stable selection. The plasmid encoding the GlyAla mutant (CMVIR3del.E1) attained a maximum copy number of only 4 per cell in the first month (20 percent of the CMVRaji plasmids). The copy number in these transformants remained relatively stable until the third month post-transfection, and were undetectable by the fourth month. In contrast, the maximum copy number of the CMVRaji.E1 plasmids reached about 24 per cell 2 months post-transfection, while the CMVB95.8.E1 plasmids peaked at about 16 per cell, and not until the third month. The relative copy number of both plasmids declined thereafter, such that by 6 months in continuous culture the B95.8 plasmids were undetectable while approximately 10 percent of the maximum amount (about 2 copies per cell) of the CMVRaji.E1 episomes were still present (**Figure 9A & B**). The eventual decline in episome copy number over time could reflect gradual inactivation of the CMV promoter by CpG methylation, resulting in decreased EBNA1 expression and eventual loss of the episomes irrespective of the EBNA1 isoform encoded on these plasmids. The CMV promoter used in these experiments has approximately 36 CpG sites. This particular viral promoter has been utilized in many integrative gene delivery systems and has been shown to be rapidly inactivated *in vitro* and especially *in vivo* (Palmer et al., *Blood* **73**, 438 (1989); Scharfmann et al., *Proc. Natl. Acad. Sci. USA* **88**, 4626 (1991).). Alternatively, the relatively abrupt loss of episomal DNA occuring over time could be due to EBNA-1 toxicity, and/or slow growth of human B cells expressing EBNA-1. Theoretical models can explain the "sudden" plasmid loss, a phenomenon extensively studied in bacteria (Summers, *Trends in Biotechnology* **9**, 273 (1991)). Various factors have been shown to alter the shape of the curve of such a "sudden loss rate", including cell growth and transgene toxicity. If so, the increased loss of episomes in the presence of the IR3-deleted EBNA-1 could be due to a more pronounced toxic effect of this mutant protein on cell metabolism.

Since the coding region of the EBNA1 truncation mutant was derived from the wild-type B95.8 EBNA1 (Yates, *supra* 1985), the differential plasmid stability observed between these two isoforms implies a role for the GlyAla domain. Interestingly, the increased rate of episomal loss in the presence of the GlyAla deletion EBNA-1 mutant was also observed in EBV positive human B cell HH514 expressing an endogenous EBNA-1 (**Fig. 8D**). Hence, such a negative trans-dominant interference by the deleted EBNA-1 isoform supports the cooperative role of this protein on OriP activation. Indeed, EBNA-1 binds as a dimer to its cognate sequence and mutiple binding on OriP are required for episomal persistence (Yates, *supra* 1996; Wysokenski et al., *supra* 1989; Yates, *supra* 1985). It is difficult to predict how the GlyAla domain influences EBNA1 activity, since most of the biochemical analyses of the EBNA1 protein were deduced from experiments using GlyAla truncated forms of EBNA1. It has been documented that EBNA1 mediates the interaction between the FR and the DS element of OriP (Frappier et al., *supra* 1991; Su et al., *supra* 1991; Middleton and Sugden, *supra* 1992). One possible explanation is that the plasmid segregation function of EBNA1 could be affected through a compromised linkage of the amino terminus to the carboxyl terminus of EBNA1 due to a reduction in the GlyAla domain. The experiments which located the DNA linking domains on EBNA1 were performed using GlyAla deletion mutants, and no comparative analysis of the linking function (or efficiency) of normal versus truncated EBNA1 proteins in long-term plasmid maintenance assays has yet been reported. The presence or not of the GlyAla domain may also affect the affinity or interaction of EBNA1 with an unidentified cellular factor involved in the segregation of DNA during mitosis.

The reasons for the difference in functional kinetics between the two wild-type EBNA1s from Raji and B95.8 are less clear. The partial removal of the GlyAla domain in Raji EBNA1 (approx 100 amino acids) may stimulate its function in long-term plasmid maintenance. Not only is the GlyAla domain of the EBNA1 protein from Raji approximately 100 amino acids shorter than the B95.8 derivative, it also has 14 amino acid differences in the C-terminus where the DNA binding and dimerization domains are located (Table 1). For comparison, the amino acid differences of the EBNA1 protein derived from an EBV-positive nasopharyngeal carcinoma (NPC)

were also included (Snudden et al., *Oncogene* **10**, 545 (1995)). According to a detailed mutational analysis of the functional domains of the B95.8 EBNA1 (Chen et al., *supra* 1993), most of the amino acid changes in the Raji EBNA1 are in regions important for EBNA1 activity. Of potential importance is the finding of a change in position 471 from a glutamine residue in the B95.8 EBNA1 to a glutamic acid residue in the Raji EBNA1. This residue is located in one of three subdomains within the 28 amino acid region that abolished DNA binding when deleted.

Role of EBNA1 threshold on long-term episome persistence and transgene expression. These results also indicate a potential minimum threshold level of EBNA1 protein necessary to establish extrachromosomal maintenance of oriP-based plasmids. The relative amount of the B95.8 EBNA1 protein expressed after 5 months (Figure 3B) did not correlate with the copy number of episomes in these cells (**Figure 8A & 9A**). Specifically, the relative amount of the B95.8 EBNA1 protein shown in **Figure 8B** (lane 6) was estimated to be approximately one tenth the Raji EBNA1 protein (lane 7), yet the relative difference in copy number between the two plasmids was less than two-fold (**Figure 9B**, lanes 14 and 15). It has been reported that EBV copy number does not correlate with the amount of EBNA1 in the cell nucleus. Sternas et al (*J. Virol.* **64**, 2407 (1990)) estimated in EBV positive B cell lines that the number of EBNA1 molecules per cell ranged between 25,000 to 44,000 (less than 2-fold difference), while the number of EBV DNA copies ranged between 10 and 400 (40-fold difference). Conversely, these data show that a large fluctuation in the level of EBNA1 protein does not significantly affect the copy number of episomes (an approximate 10 fold difference in EBNA1 protein, yet only a 2 fold plasmid copy number difference). It should be noted, though, that the EBV-positive cells used in the experiments of Sternas et al. are qualitatively different than the EBV-negative cells used in these experiments. Once the number of EBNA1 molecules falls below a certain threshold, episomes probably integrate as indicated by the absence of detectable episomes after 4 months for CMVIR3del.E1 and after 6 months for the CMVB95.8.E1 transformants (**Figure 9A**, lane 17). In support, it was observed that expression of the EBNA1 protein from a weak cellular promoter (relative to CMV) was insufficient to establish the same OriP/EBNA1 episomes in DG75 or BL30, another EBV negative BL cell line (unpublished data). Altogether these results imply

that a threshold level of EBNA1 expression is required in human cells to establish episomal maintenance of transfected OriP-based plasmids.

The experiments presented here indicate that the absence of the GlyAla domain of EBNA1 influences the ability of this protein to function adequately at OriP. This subtle influence becomes evident in long-term plasmid maintenance and transgene expression assays. It was observed that a novel EBNA1 isoform isolated from Raji EBV, may be functionally superior to the standard B95.8 derived EBNA1 in plasmid persistence and transgene expression. It was shown that transgenes will be expressed at high levels for prolonged periods provided that OriP/EBNA1 plasmids remain episomal. Several observations were made from the fine comparison of the relative changes in beta-gal activity (**Fig. 8C**) and copy number (**Fig. 4B**) as a function of time. First, both of these endpoints became drastically reduced after long-term cultures reflecting a common inactivating event, irrespective of the plasmid. Second, an accelerated decrease occured for both parameters with the IR3-deleted vector. Third, the relative levels of beta-gal activity and episomal copy numbers did not always appeared to be directly proportional, particularly at early time points. The EBNA-1-mediated transcriptional activation of the SV40 promoter driving the beta-gal expression may respond differentially to variable levels of EBNA-1 protein (**Fig. 7B & 8B**) than replication initiation at OriP. Alternatively, squelching on the SV40 promoter, i.e. titration of a limiting transcriptional factor, may saturate the level of transgene expression at high episomal copy number. At later time points however, both values were decreased by 10 to 100 fold, while being maintained at the highest levels in cells expressing the EBNA-1 Raji (**Fig. 8C-D & 9B**).

In summary, the main conclusions of this work are i) the relative instability of an IR3-deleted EBNA-1 and ii) the enhanced stability of the EBNA-1 Raji for long-term maintenance of OriP-based plasmids in human cells. These findings are relevant to gene delivery protocols designed for persistence of extrachromosomal plasmids. Current OriP/EBNA-1 plasmids require the selection because of low transfection efficencies and relative instability in dividing human cells. Obviously, *ex vivo* gene therapy protocols which are based on the *in vitro* selection of stably transduced cells, can use the hygromycin selection. In addition, there are *in vivo*-selectable genes which could replace the hygromycin as dominant marker drug (Summers, *Trends in*

Biotechnology 9, 273 (1991)). The development of episomal gene delivery vectors that can deliver sustained gene expression, without potentially harmful integration, would be advantageous in treating patients with various and common genetic deficiencies via gene therapy.

The foregoing is illustrative of the present invention, and is not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.

What is Claimed is:

1. A recombinant plasmid useful for the production of large-insert episomes in mammalian cells, comprising:

a lymphotrophic herpes virus segment which (a) contains an origin of plasmid replication (oriP) and a heterologous origin of bacterial replication, and (b) is maintained as an episome in both bacterial and mammalian host cells; and

a heterologous insert segment linked to said lymphotrophic herpes virus segment, said heterologous insert segment having a length of at least about 50 kilobases.

2. A recombinant plasmid according to claim 1, wherein said lymphotrophic herpes virus segment is capable of producing infectious virions in a suitable host cell, and wherein said lymphotrophic herpes virus segment has regions deleted so that said recombinant plasmid retains the capability of producing infectious virions in said host cell.

3. A recombinant plasmid according to claim 1, wherein said heterologous insert segment has a length of at least about 100 kilobases.

4. A recombinant plasmid according to claim 1, wherein said heterologous insert segment has a length of at least about 150 kilobases.

5. A recombinant plasmid according to claim 1, said heterologous insert segment including a centromere operable in said host cell.

6. A recombinant plasmid according to claim 1, wherein said lymphotrophic herpes virus segment comprises an Epstein-Barr virus segment.

7. A recombinant plasmid according to claim 1, wherein said lymphotrophic herpes virus segment has regions deleted which render said recombinant plasmid capable of producing infectious virions only in a host cell containing helper

sequences.

8. A method for transforming mammalian cells. said method comprising transfecting a mammalian cell with a recombinant plasmid, said recombinant plasmid comprising:

a lymphotrophic herpes virus segment which (a) contains an origin of plasmid replication (oriP) and a heterologous origin of bacterial replication, and (b) is maintained as an episome in both bacterial and mammalian cells; and

a heterologous insert segment linked to said lymphotrophic herpes virus segment, said heterologous insert segment having a length of at least about 100 kilobases.

9. A method according to claim 8, wherein said lymphotrophic herpes virus segment is capable of producing infectious virions in a suitable host cell, and wherein said lymphotrophic herpes virus segment has regions deleted so that said recombinant plasmid retains the capability of producing infectious virions in said host cell.

10. A method according to claim 8, wherein said mammalian cells are grown as a monolayer in in vitro cell culture, and wherein said transfecting step is carried out by lipofection.

11. A method according to claim 8, wherein said mammalian cell is a B-lymphoblastoid cell.

12. A method according to claim 8, wherein said mammalian cell is a fusion of a mammalian epithelial cell and a mammalian B-lymphoblastoid cell.

13. A method according to claim 8, wherein said mammalian cell is an epithelial cell.

14. A method according to claim 8, said heterologous insert segment including a centromere operable in said mammalian cell.

15. A method according to claim 8, wherein said mammalian cell is capable of producing infectious virions from said recombinant plasmid.

16. A method according to claim 8, wherein said mammalian cell is incapable of producing infectious virions from said recombinant plasmid.

17. A transformed mammalian cell containing a recombinant plasmid, said recombinant plasmid comprising:

a lymphotrophic herpes virus segment which (a) contains an origin of plasmid replication (oriP) and a heterologous origin of bacterial replication, and (b) is maintained as an episome in both bacterial and mammalian cells; and

a heterologous insert segment linked to said lymphotrophic herpes virus segment, said heterologous insert segment having a length of at least about 50 kilobases.

18. A transformed mammalian cell according to claim 17, wherein said lymphotrophic herpes virus segment is capable of producing infectious virions in a suitable host cell, and wherein said lymphotrophic herpes virus segment has regions deleted so that said recombinant plasmid retains the capability of producing infectious virions in said suitable host cell.

19. A transformed mammalian cell according to claim 17, said heterologous insert segment including a centromere operable in said mammalian cell.

20. A transformed mammalian cell according to claim 17, wherein said cell is grown as a monolayer in in vitro cell culture.

21. A transformed mammalian cell according to claim 17, wherein said cell is a human cell.

22. A transformed mammalian cell according to claim 17, wherein said cell is capable of producing infectious virions from said recombinant plasmid.

23. A transformed mammalian cell according to claim 17, wherein said cell is a B-lymphoblastoid cell.

24. A transformed mammalian cell according to claim 17, wherein said cell is a fusion of a mammalian epithelial cell and a mammalian B-lymphoblastoid cell.

25. A transformed mammalian cell according to claim 17, wherein said cell is an epithelial cell.

26. A transformed mammalian cell according to claim 17, wherein said heterologous insert segment has a length of at least about 100 kilobases.

27. A transformed mammalian cell according to claim 17, wherein said heterologous insert segment has a length of at least about 150 kilobases.

28. A transformed mammalian cell according to claim 17, wherein said lymphotrophic herpes virus segment comprises an Epstein-Barr virus segment.

29. A large insert DNA library comprising a plurality of transformed mammalian cells, each of said transformed mammalian cells containing a recombinant plasmid, said recombinant plasmid comprising:

a lymphotrophic herpes virus segment which (a) contains an origin of plasmid replication (oriP) and a hetrologous origin of bacterial replication, and (b) is maintained as an episome in both bacterial and mammalian host cells; and

a heterologous insert segment linked to said lymphotrophic herpes virus segment, said heterologous insert segment having a length of at least about 50 kilobases and comprising a member of said DNA library.

30. A large-insert DNA library according to claim 29, said lymphotrophic herpes virus segment having regions deleted so that said recombinant plasmid retains the capability of producing infectious virions in a suitable host cell.

31. A large-insert DNA library according to claim 29, wherein said library is a

human genomic DNA library, and wherein said mammalian cells are human cells.

32. A large-insert DNA library according to claim 29, wherein said lymphotrophic herpes virus segment is an Epstein-Barr virus segment, and wherein said mammalian cells are human B-lymphoblastoid cells.

33. A large-insert DNA library according to claim 29, wherein said library is a partial genomic DNA library.

34. A large-insert DNA library according to claim 29, wherein said library is a complete genomic DNA library.

35. A large-insert DNA library comprising a plurality of infectious lymphotrophic herpes virus virions, each of said virions containing a recombinant DNA molecule, said recombinant DNA molecule comprising:

a lymphotrophic herpes virus segment which is capable of infecting mammalian cells, is maintained as an episome therein, and produces infectious virions in a suitable host; and

a heterologous insert segment linked to said lymphotrophic herpes virus segment, said heterologous insert segment having a length of at least 50 kilobases and comprising a member of said DNA library;

said lymphotrophic herpes virus segment having regions deleted so that said recombinant DNA sequence retains the capability of producing infectious virions in a suitable host;

said lymphotrophic herpes virus segment including an origin of plasmid replication (oriP), a heterologous origin of bacterial replication, a lytic origin of replication (oriLyt), and at least one long terminal repeat region (TR).

36. A large-insert DNA library according to claim 35, wherein said library is a human genomic DNA library.

37. A large-insert DNA library according to claim 35, wherein said ymphotrophic herpes virus segment is an Epstein-Barr virus segment.

38. A large-insert DNA library according to claim 35, wherein said library is a partial genomic DNA library.

39. A large-insert DNA library according to claim 35, wherein said library is a complete genomic DNA library.

40. A recombinant plasmid useful for the production of large-insert episomes in mammalian cells, comprising:

an Epstein-Barr virus segment containing an origin of plasmid replication (oriP), a lytic origin of replication (oriLyt), a heterologous origin of bacterial replication and a fused long terminal repeat region (TR); and

a heterologous insert segment linked to said Epstein-Barr virus segment, said heterologous insert segment having a length of at least 50 kilobases.

41. A method for transforming mammalian cells, said method comprising transfecting a mammalian cell with a recombinant plasmid of claim 40.

42. A transformed mammalian cell containing a recombinant plasmid according to claim 40.

43. A large-insert DNA library comprising a plurality of transformed mammalian cells, each of said transformed mammalian cells containing a recombinant plasmid according to claim 40.

44. An infectious lymphotrophic herpes virus virion containing a recombinant DNA molecule, said recombinant DNA molecule comprising:

a lymphotrophic herpes virus segment which is capable of infecting mammalian cells, is maintained as an episome therein, and produces infectious virions in a suitable host; and

a heterologous insert segment linked to said lymphotrophic herpes virus segment, said heterologous insert segment having a length of at least 50 kilobases;

said lymphotrophic herpes virus segment having regions deleted so that said recombinant DNA sequence retains the capability of producing infectious virions in a suitable host;

said lymphotrophic herpes virus segment including an origin of plasmid replication (oriP), a heterologous origin of bacterial replication, a lytic origin of replication (oriLyt), and at least one long terminal repeat region (TR).

45. An infectious lymphotrophic herpes virus virion according to claim 44, wherein said lymphotrophic herpes virus segment is an Epstein-Barr virus segment.

46. An infectious lymphotrophic herpes virus virion according to claim 44, wherein said heterologous insert segment has a length of at least 100 kilobases.

47. An infectious lymphotrophic herpes virus virion according to claim 44, wherein said heterologous insert segment has a length of at least 150 kilobases.

48. An infectious lymphotrophic herpes virus virion according to claim 44, said heterologous insert segment including a centromere operable in a mammalian cell.

49. A method of introducing a DNA of interest into a mammalian subject, comprising:

directly or indirectly administering a plasmid according to claim 1 to said subject in an amount effective to introduce said DNA of interest.

50. A transgenic non-human mammal comprising cells containing a plasmid according to claim 1.

51. A method of producing a heterologous protein in a host cell, comprising the steps of:

transfecting said host cell with a plasmid according to claim 1, wherein said heterologous insert encodes and expresses said heterologous protein in said host cell; and

expressing said heterologous protein in said host cell.

52. A method according to claim 51, wherein said host cell is selected from the group consisting of bacterial cells and mammalian cells.

53. A recombinant plasmid according to claim 1, said plasmid further comprising a copy of an EBV Nuclear Antigen-1 (EBNA-1) gene that can be functionally expressed in a mammalian cell.

54. A recombinant plasmid according to claim 53, wherein said EBNA-1 gene includes a partial IR3 domain deletion.

55. A recombinant plasmid according to claim 53, wherein said EBNA-1 gene is the Raji EBNA-1 gene.

56. A method according to claim 8, said plasmid further comprising a copy of an EBV Nuclear Antigen-1 (EBNA-1) gene that can be functionally expressed in a mammalian cell.

57. A method according to claim 56, wherein said EBNA-1 gene includes a partial IR3 domain deletion.

58. A recombinant plasmid according to claim 57, wherein said EBNA-1 gene is the Raji EBNA-1 gene.

59. A cell according to claim 17, said plasmid further comprising a copy of an EBV Nuclear Antigen-1 (EBNA-1) gene that can be functionally expressed in a mammalian cell.

60. A cell according to claim 59, wherein said EBNA-1 gene includes a partial IR3 domain deletion.

61. A cell according to claim 59, wherein said EBNA-1 gene is the Raji EBNA-1 gene.

62. A library according to claim 29, said plasmid further comprising a copy of an EBV Nuclear Antigen-1 (EBNA-1) gene that can be functionally expressed in a mammalian cell.

63. An isolated nucleic acid encoding an EBV Nuclear Antigen-1 (EBNA-1) gene that has a partial IR3 domain deletion, wherein said IR3 region is from 300 to 700 nucleotides in length.

64. An isolated nucleic acid according to claim 63, wherein said EBNA-1 gene is the Raji EBNA-1 gene.

65. A vector containing an isolated nucleic acid according to claim 63.

66. A plasmid containing an isolated nucleic acid according to claim 63.

67. A method of introducing a DNA of interest into a mammalian subject, comprising:

directly or indirectly administering virions according to claim 44 to said subject in an amount effective to introduce said DNA of interest.

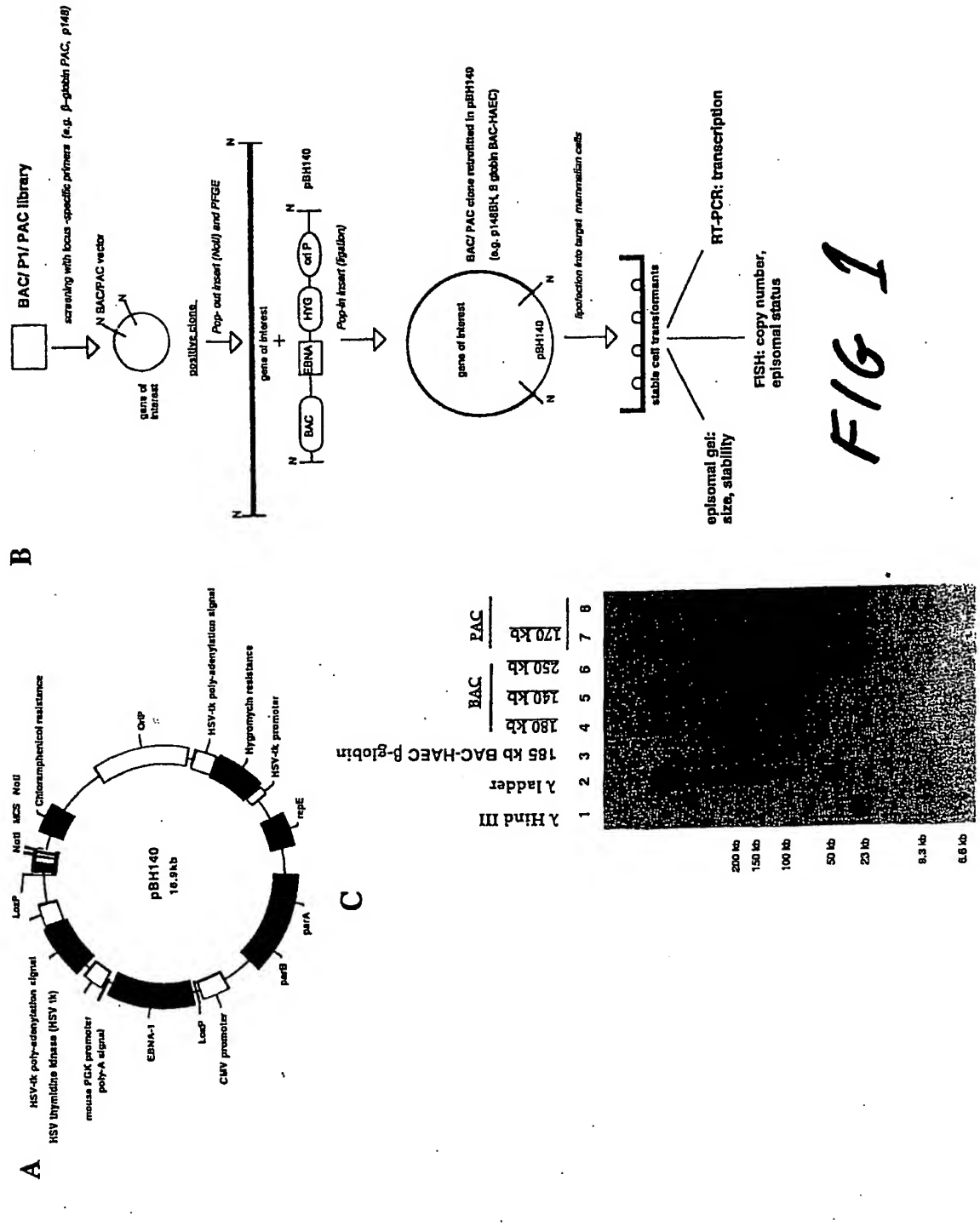
68. A transgenic non-human mammal comprising cells transfected with a virion according to claim 44.

69. A method of producing a heterologous protein in a host cell, comprising the steps of:

transfecting said host cell virions according to claim 44, wherein said heterologous insert encodes and expresses said heterologous protein in said host cell; and

expressing said heterologous protein in said host cell.

70. A method according to claim 69, wherein said host cell is selected from the group consisting of bacterial cells and mammalian cells.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gene of interest
positive clone
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gene of interest
+
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(e.g. p148BH, β globin BAC-HAEC)
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stable cell transformants
episomal gel:
size, stability
RT-PCR: transcription
FISH: copy number,
episomal status
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λ ladder
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FIG 1

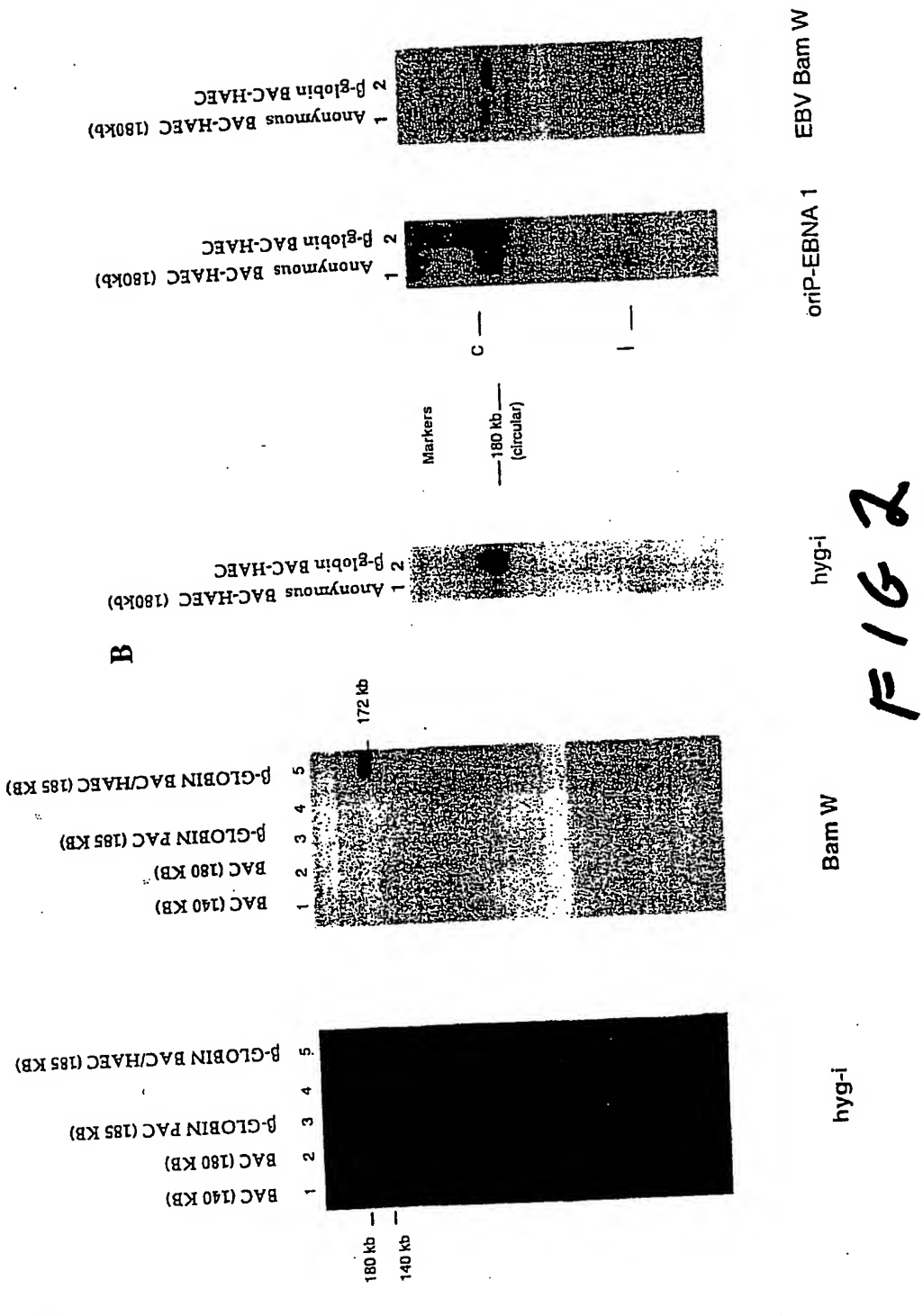
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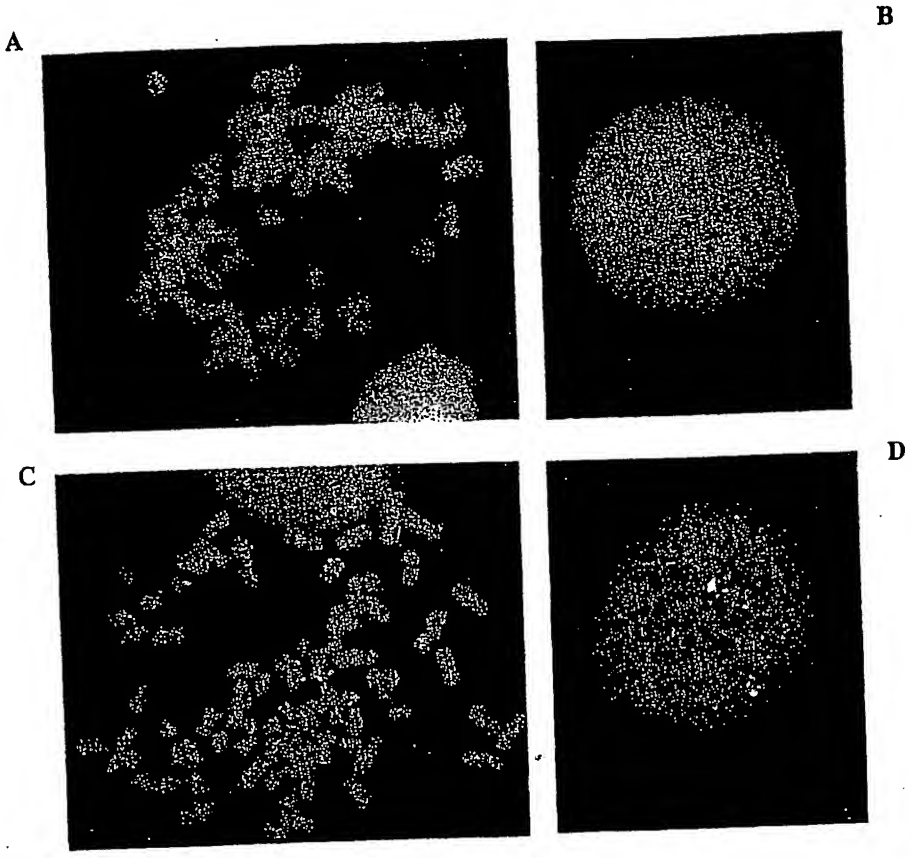
FIG 2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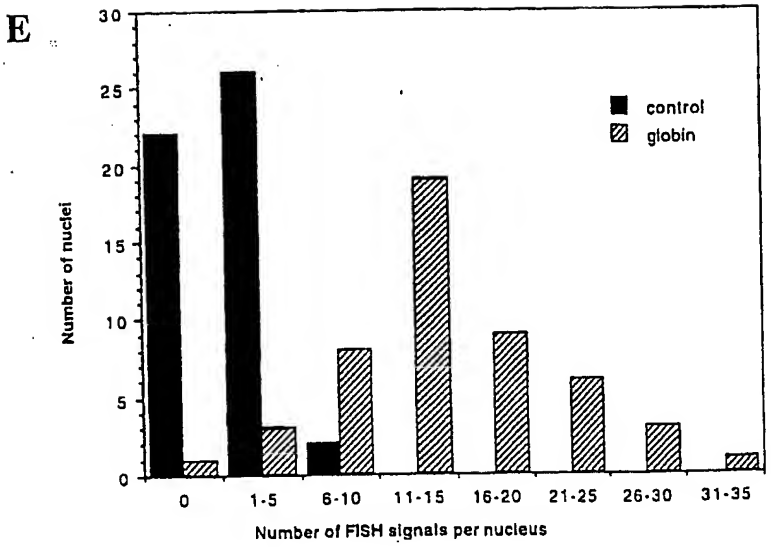
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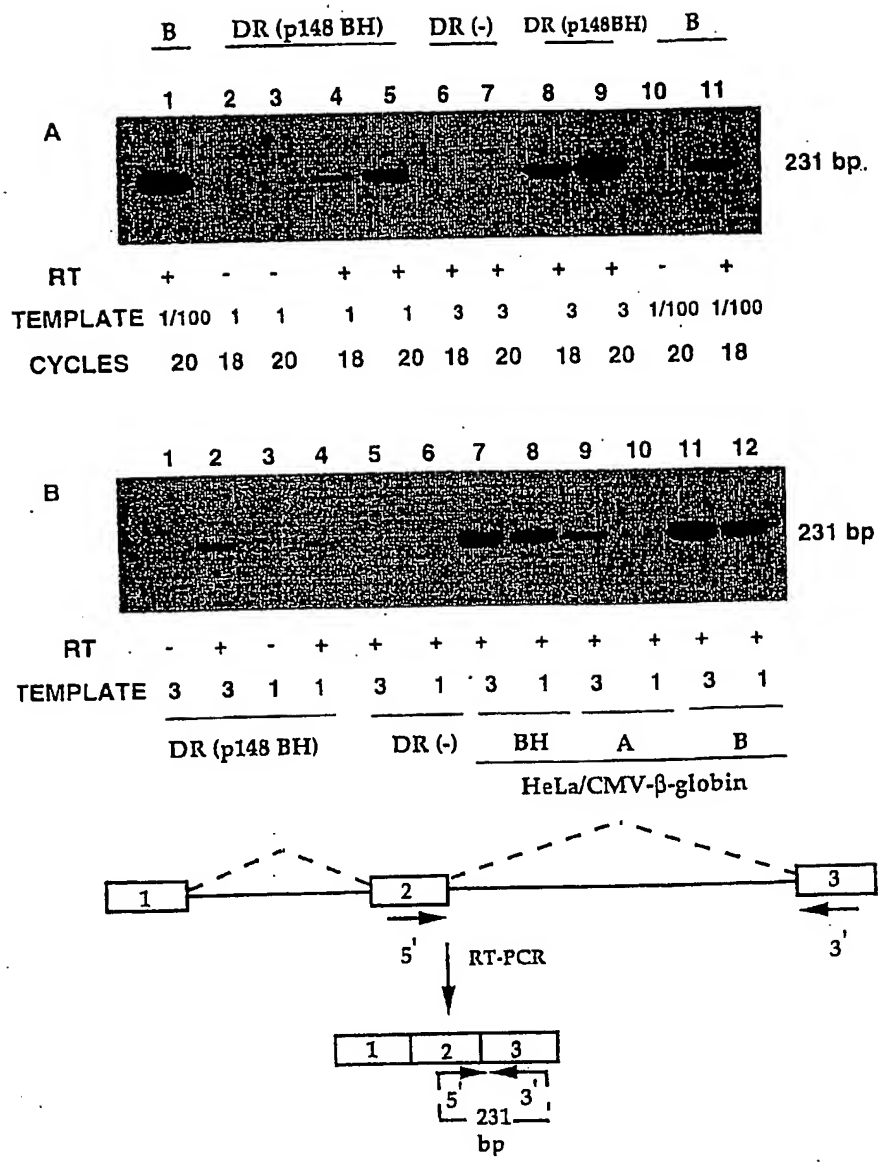
Number of FISH signals per nucleus

FIG 3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BH
A
B
HeLa/CMV-β-globin
1
2
3
5'
RT-PCR
3'
1 2 3
5'
3'
231
bp

FIG 4

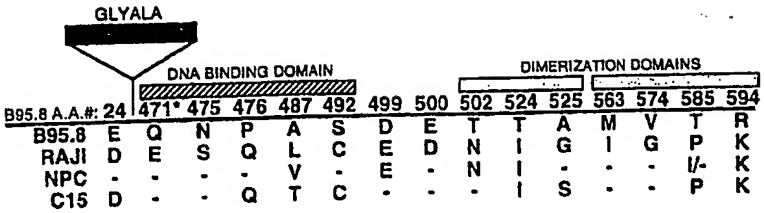
GLYALA
DNA BINDING DOMAIN
DIMERIZATION DOMAINS
B95.8 A.A.#: 24 471* 475 476 487 492 499 500 502 524 525 563 574 585 594
B95.8 E Q N P A S D E T T A M V T R
RAJI D E S Q L C E D N I G I G P K
NPC - - - - V - E - N I - - - I/- K
C15 D - - Q T C - - - I S - - P K

FIG 5

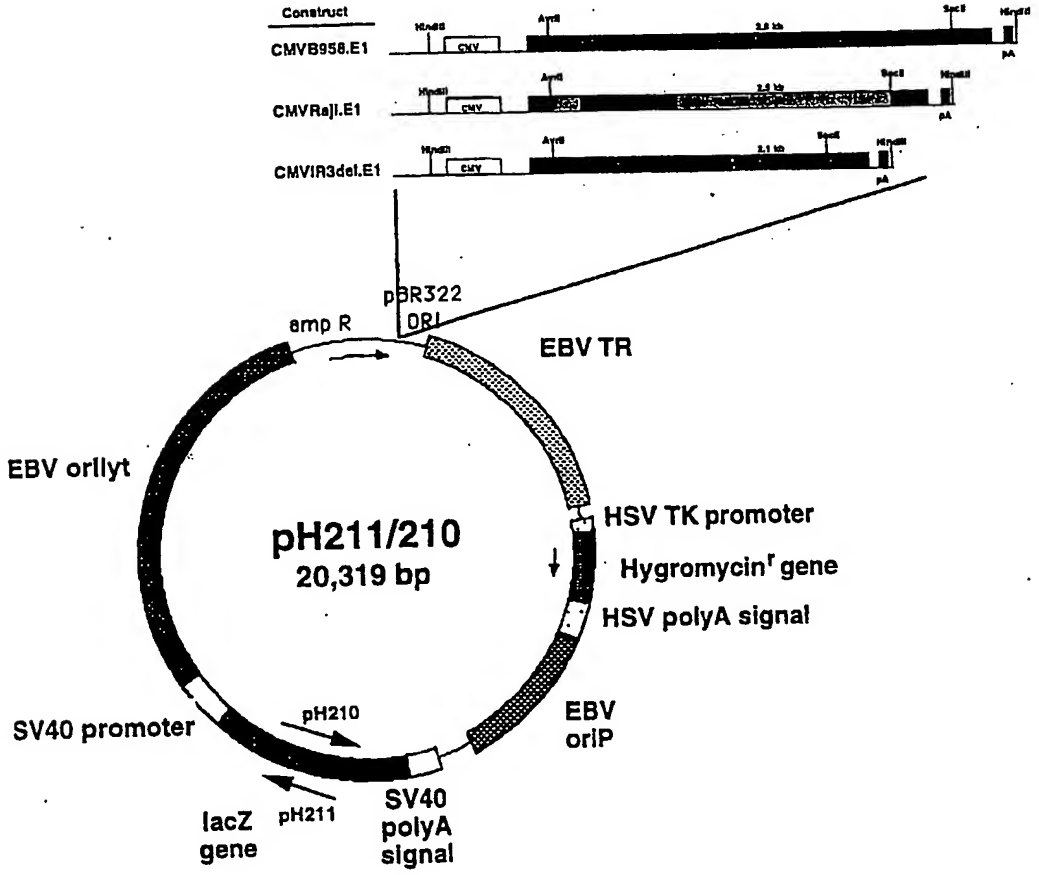
Construct
CMVB958.E1
CMVRajI.E1
CMVIR3del.E1
pBR322
ori
amp R
EBV TR
EBV orilyt
pH211/210
20,319 bp
HSV TK promoter
Hygromycin^r gene
HSV polyA signal
EBV
oriP
SV40 promoter
pH210
lacZ
gene
pH211
SV40
polyA
signal

FIG 6

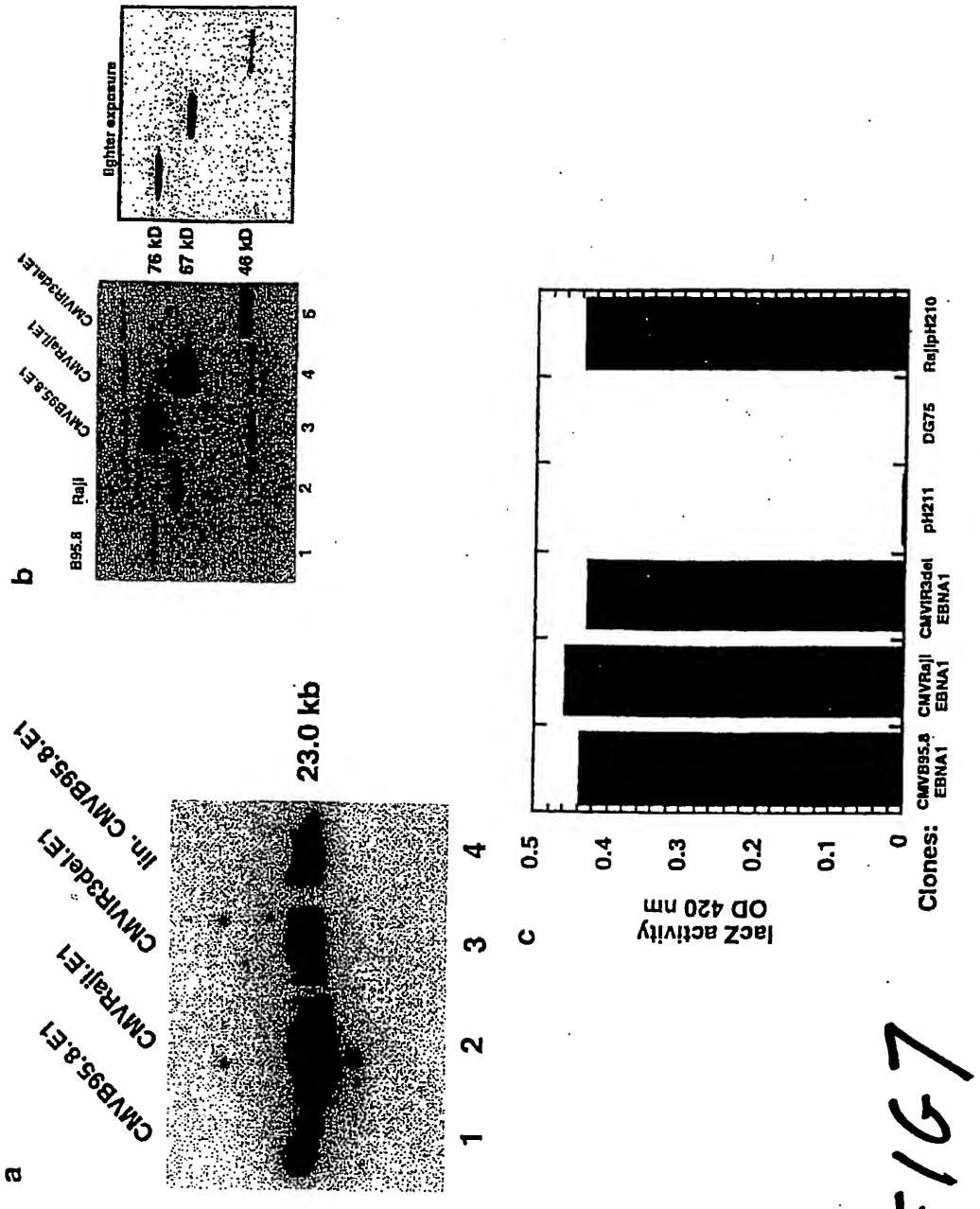
a
CMVB95.8.E1
CMVRaji.E1
CMVIR3del.E1
lin. CMVB95.8.E1
1 2 3 4
23.0 kb
b
B95.8
Raji
CMVB95.8.E1
CMVRaji.E1
CMVIR3del.E1
1 2 3 4 5
76 kD
67 kD
46 kD
lighter exposure
c
lacZ activity
OD 420 nm
0 0.1 0.2 0.3 0.4 0.5
Clones:
CMVB95.8 EBNA1
CMVRaji EBNA1
CMVIR3del EBNA1
pH211
DG75
RajIpH210

FIG 7

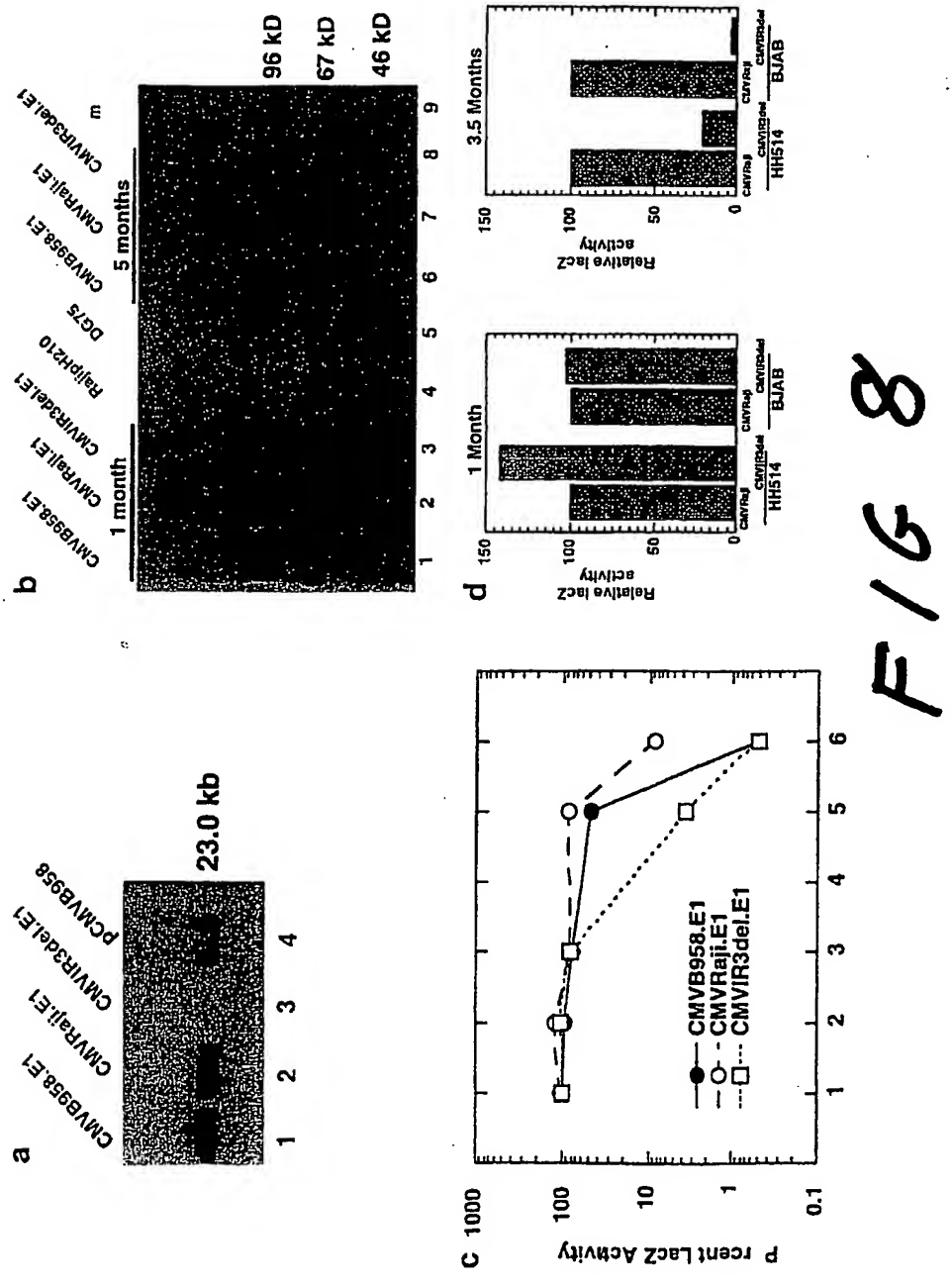
a
CMVB958.E1
CMVRaji.E1
CMVIR3del.E1
pCMVB958
23.0 kb
1 2 3 4
b
CMVB958.E1
CMVRaji.E1
CMVIR3del.E1
Raji pH2.10
DG75
CMVB958.E1
CMVRaji.E1
CMVIR3del.E1
m
1 month
5 months
96 kD
67 kD
46 kD
1 2 3 4 5 6 7 8 9
c
Percent LacZ Activity
1000
100
10
1
0.1
CMVB958.E1
CMVRaji.E1
CMVIR3del.E1
1 2 3 4 5 6
d
1 Month
3.5 Months
Relative lacZ activity
150
100
50
0
CMVRaji
CMVIR3del
HH514
BJAB

FIG 8

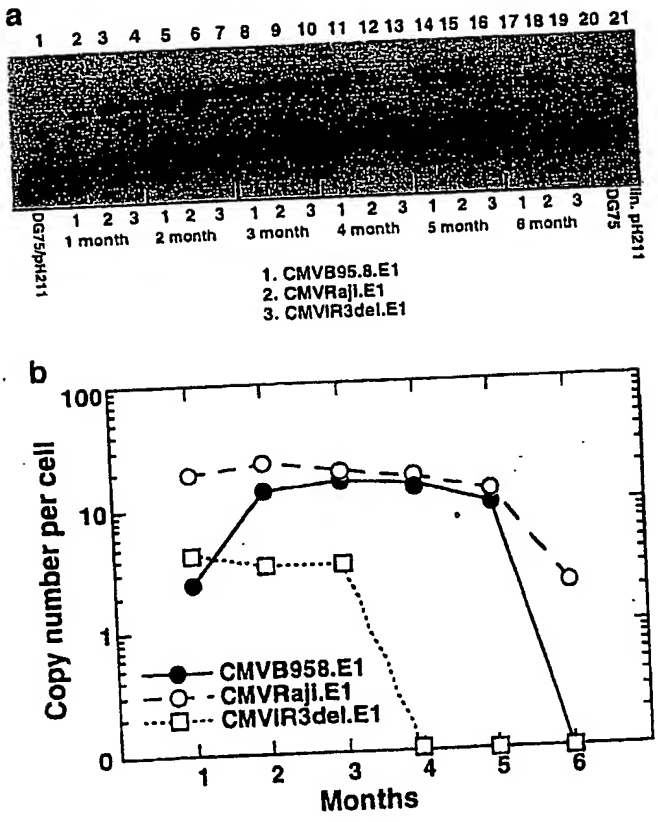
a
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
DG75/pHt211
1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3
1 month
2 month
3 month
4 month
5 month
6 month
DG75
lin. pHt211
1. CMVB95.8.E1
2. CMVRaji.E1
3. CMVIR3del.E1
b
Copy number per cell
100
10
1
0
CMVB958.E1
CMVRaji.E1
CMVIR3del.E1
1 2 3 4 5 6
Months

FIG 9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/19468

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12N 15/11, 15/63, 15/85, 15/86, 7/00; C07K 14/00
US CL : 536/23.1; 435/320.1, 325, 235.1; 530/350
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.1; 435/320.1, 325, 235.1; 530/350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST 1.2, STN/CAS: MEDLINE, SCISEARCH, CAPLUS, BIOSIS
Search terms: Episome, Herpes, Ebstein virus, Transgenic, Therapy.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PESANO.et. al. Herpesvirus papio contains a plasmid origin of replication that acts in cis interspecies with Epstein-Barr virus trans-acting function. Journal of Virology. December 1986. Vol. 60. No. 3. pages 159-1162, see entire document.	1-66
A	GELLER.et.al.. An efficent deletion mutant packaging system for defective herpes simplex virus vectors: Potential applications to human gene therapy and neuronal phsiology. Proceedng of the National Acadmy of Sciences USA. November 1990. Vol. 87. No. 22. pages 8950-8954, see entire document	1-66

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"B"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 04 NOVEMBER 1999	Date of mailing of the international search report 03 FEB 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer SUMESH KAUSHAL Telephone No. (703) 305-6838

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/19468

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category°	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GELLER. et.al. Infection of cultured central nervous system neurons with a defective herpes simplex virus 1 vector results in stable expression of Escherichia coli. beta-galactosidase. February 1990. Vol. 87. No.3. pages 1149-1153. see entire document.	1-63
Y	US 5,194,601 A (SUGDEN et al) 16 March, 1993, see entire document	1-48
XY	US 5,275,942 A (VOS) 04 January 1994, see entire document.	X 1-48 Y 51-52, 69-70
Y	US 5,707,830 A (CALOS) 13 January 1998, col.4 line 30-64, col 14. line 30-42. col. 36 line 61.	53-66

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

Document AL2
Appl. No. 10/035,216

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification: C12N 15/10, A01K 67/027, C12N 5/06, C12N 15/86, C12Q 1/68	A1	(11) International Publication Number:	WO 00/28016
		(43) International Publication Date:	18 May 2000 (18.05.2000)

(21) International Application Number: PCT/US98/24029

(22) International Filing Date: 10 November 1998 (10.11.1998)

(60) Parent Application or Grant
UNIVERSITY OF ROCHESTER [/]; (). ZAUDERER, Maurice [/]; (). STEFFE, Eric, K. ; ().

Published

(54) Title: T CELLS SPECIFIC FOR TARGET ANTIGENS AND METHODS AND VACCINES BASED THEREON

(54) Titre: LYMPHOCYTES T SPECIFIQUES D'ANTIGENES CIBLE, VACCINS PREPARES A PARTIR DESDITS LYMPHOCYTES, ET METHODES ASSOCIEES

(57) Abstract

The present invention relates to novel methods for the identification of antigens recognized by cytotoxic T cells (CTLs) and specific for human tumors, cancers, and infected cells, and the use of such antigens in immunogenic compositions or vaccines to induce regression of tumors, cancers, or infections in mammals, including humans. The invention encompasses methods for induction and isolation of cytotoxic T cells specific for human tumors, cancers and infected cells, and for improved selection of genes that encode the target antigens recognized by these specific T cells. The invention also relates to differential display methods that improve resolution of, and that reduce the frequency of false positives of DNA fragments that are differentially expressed in tumorous, cancerous, or infected tissues versus normal tissues. The invention further relates to the engineering of recombinant viruses as expression vectors for tumor, cancer, or infected cell-specific antigens.

(57) Abrégé

La présente invention concerne de nouvelles méthodes d'identification d'antigènes, reconnus par les lymphocytes T cytotoxiques (CTL), et spécifiques des tumeurs, cancers et autres cellules infectées chez l'homme, ainsi que l'utilisation de ces antigènes dans des compositions ou vaccins immunogènes pour induire une régression des tumeurs, cancers, ou infections chez les mammifères, notamment chez l'homme. L'invention concerne donc des méthodes permettant d'induire et d'isoler des lymphocytes T cytotoxiques spécifiques des tumeurs, cancers et autres cellules infectées chez l'homme, et de sélectionner de façon plus précise les gènes codant pour les antigènes cible reconnus par ces lymphocytes T spécifiques. L'invention concerne également des méthodes d'affichage différentiel améliorant la résolution des fragments d'ADN et réduisant la fréquence des faux positifs des fragments d'ADN exprimés de façon différentielle dans des tissus tumoraux, cancéreux ou infectés et dans les tissus normaux. L'invention concerne enfin la mise au point par génie génétique de virus de recombinaison faisant office de vecteurs d'expression pour les antigènes spécifiques des tumeurs, cancers et autres cellules infectées.